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PRINCIPAL INVESTIGATOR: Rafael A. Fridman, Ph.D.

CONTRACTING ORGANIZATION: Wayne State University
Detroit, Michigan 48202

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13. ABSTRACT (Maximum 200 words) Metastasis is the major cause of death in breast cancer patients. To metastasize, breast cancer cells must degrade extracellular matrices (ECM) using specialized ECM-degrading proteases including matrix metalloproteinases (MMPs) and cathepsins. The purpose of these studies was to understand the mechanisms regulating the expression, cellular localization and activation of MMP-2, MMP-9 and cathepsin B in breast epithelial and fibroblast cells. We have found that breast cancer cells can be induced to express latent MMP-9 (pro-MMP-9) by interactions with platelets and exposure to growth factors and phorbol ester. Induction of pro-MMP-9 expression results in the localization of a TIMP-free enzyme on the cell surface, a process that is partly mediated by the $\alpha 2(\text{IV})$ chain of collagen IV. We have found that pro-MMP-2 is activated by MT1-MMP on the surface of breast fibroblasts seeded in a collagen I matrix by inducing MT1-MMP expression. Binding of MMP-2 to the cell surface of breast cancer cells is dependent on cell density. We showed that MMP-2 could in turn activate surface-bound pro-MMP-9. Studies with cathepsin B showed a specific localization of enzyme on the surface of breast cancer cells. Taken together, these studies provided novel information on the molecular mechanisms that contribute to breast cancer metastasis.					
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FOREWORD

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5. INTRODUCTION

Tumor metastasis is the major cause of treatment failure in breast cancer patients. Numerous studies have shown that metastasis formation depends on the ability of the tumor cells to invade basement membranes and connective tissue matrices in a process involving a specialized group of enzymes capable of degrading extracellular matrix (ECM) components (1, 2, 3). Studies with various enzyme systems including the serine proteases (4), the matrix metalloproteinases (MMPs) (5, 6, 7, 8, 9, 10) and the cathepsins (11) have shown that the degradation of ECM during tumor cell invasion is the result of a collaborative action between enzymes, enzyme receptors and enzyme inhibitors produced by both the tumor and the stroma cells. For example, tumor cells may utilize stromal enzymes during their invasion of ECM by binding enzymes to specific cell surface receptors (12) and/or by producing enzymes capable of activating stromal proteases (13). Indeed, tumor cells can induce the expression of ECM-degradative enzymes in stromal cells (14, 15) or they can induce the stroma to produce specific enzyme inhibitors (16, 17, 18).

Matrix Metalloproteinases. The MMPs are a family of highly conserved zinc-dependent proteinases capable of degrading many extracellular matrix (ECM) components (19, 20). Although all the MMPs can degrade ECM, several of them, in particular, were shown to be associated with breast cancer. These include MMP-2 (gelatinase A, 72 kDa type IV collagenase), MMP-9 (gelatinase B, 92 kDa type IV collagenase), stromelysin-3, collagenase-3 and membrane type-MMP (MT-MMP), in particular MT1-MMP (6, 8, 21, 22, 23). The MMPs, except stromelysin-3 and the MT-MMPs, are secreted in a latent form that requires activation to become proteolytically active (24). In the case of stromelysin-3, activation occurs intracellularly and it is mediated by a furin-like enzyme (25). The mechanism of MT-MMP activation is still unknown but may also involve intracellular activation mediated by furin (26). Proenzyme activation is a critical event in regulation of MMP activity and may be essential for ECM degradation during tumor cell invasion. The physiological mechanisms responsible for MMP activation in breast cancer tissues are not completely understood, but may involve the action of other proteases, including other MMPs. Previous studies have shown that MT1-MMP, an MMP present in the plasma membrane, may be the physiological activator of MMP-2 (27). Since activation of pro-MMP-2 has been shown to be induced by a collagen I gel (28) and since fibroblasts are the major producers of MT1-MMP in breast tumor (6), it is likely that the interactions of the stromal fibroblasts with collagen I may play an important role in the physiological activation of pro-MMP-2 by MT1-MMP in breast tumors.

Tissue Inhibitors of Metalloproteinases. The MMPs are all inhibited by the tissue inhibitor of metalloproteinases (TIMPs), a conserved family of low molecular weight proteins that presently includes TIMP-1, TIMP-2, TIMP-3 and TIMP-4 (29). The association of TIMP-1 and TIMP-2 with the gelatinases is unique since the inhibitors can also form a stable complex with the latent enzymes. For instance, TIMP-1 is capable of binding to pro-MMP-9 (30) whereas TIMP-2 can form a complex with pro-MMP-2 (31). This unique interaction of the TIMPs with the proenzymes may provide an additional level of regulation by preventing generation of full enzymatic activity. However, recent studies have shown a possible role of TIMP-2 on the activation of pro-MMP-2 by MT1-MMP on the cell surface (32, 33).

Epithelial-Stromal Regulation of ECM-degrading Proteases in Breast Cancer. In breast cancer, expression of MMPs has been suggested to play a role in tumor progression. Immunohistochemical studies showed elevated expression of MMP-2 and MMP-9 in the tumor cells (18, 34, 35, 36, 37). *In situ* hybridization studies, however, showed mRNA expression for enzymes and TIMP-2 in the fibroblasts around invasive tumor cell clusters (8, 10) suggesting an important role for the stromal cells in MMP expression in breast cancer. In the case of pro-MMP-2, it was speculated that the activator of this enzyme, MT1-MMP, was probably localized in the breast cancer cells. This would allow for activation of the stromal pro-MMP-2 enzyme on the surface of the tumor cells. However, in a recent study the mRNA for MT1-MMP was also localized in the stroma of breast cancer tissues (6). Interestingly, the MT1 MMP protein was found in the breast cancer cells (38). The reason for this differential localization is unknown but may suggest a differential stability of MT1-MMP protein and mRNA in epithelial and stroma cells or alternatively the interesting possibility that MT1-MMP may bind to the surface of breast cancer cells after release from the fibroblasts similarly to the proposed mechanism for the localization of MMP-2 on the surface of breast cancer cells (8). In the case of pro-MMP-9, it has also been shown that the protein is expressed on the surface of the breast cancer cells (18). However, like pro-MMP-2 and MT1-MMP, the mRNA of pro-MMP-9 is localized, in addition to the tumor cells, in the tumor stroma in particular in fibroblasts, macrophages and endothelial cells (39). This raises the question of how pro-MMP-9 and/or its active form associates with the breast epithelial cells in breast tumors. Pro-MMP-9 does not bind to MT1-MMP on the cell surface and no other specific receptor/activator for pro-MMP-9 has been reported in breast cancer cells. Therefore, the mechanisms of cell surface association of pro-MMP-9 remain unknown.

Purpose of the Studies: During the tenure of this grant we investigated the expression and cell surface localization of proteases in breast cancer cells. Our aim was to understand the mechanisms that regulate the expression and activity of proteases known to play a role in breast cancer metastasis. We concentrated on the MMP family and on cathepsin B as proposed in our original application. Studies were carried out with immortalized breast epithelial cells, breast cancer cell lines and fibroblasts derived from breast tissues. In this final report, we describe the major findings encountered during these studies.

6. BODY OF REPORT

Note: The reviewers are respectfully referred to the appendices for methodology and figures.

6.1 Activation of pro-MMP-2 by MMP-9 (Cancer Res., 55, 2548-2555, 1995)

In breast tumors, both gelatinases are known to be expressed. Whereas pro-MMP-2 is activated by MT1-MMP, the mechanism of pro-MMP-9 activation in breast cancer tissues is uncertain. We investigated whether pro-MMP-9 can be activated by MMP-2 and found that MT1-MMP-activated MMP-2 can in turn activate pro-MMP-9 to an 82-kDa active form in a process inhibited by TIMP-1 and TIMP-2. Pro-MMP-9 activation was accomplished by the two active species of MMP-2, the 62- and 45-kDa forms. The latter lacks the C-terminal domain, which is known to play a role in plasma membrane activation and in TIMP-2 inhibition. These results suggest a novel mechanism of activation of pro-MMP-9 mediated by MMP-2 that may be

localized in the surface of breast cancer cells and enhance matrix degradation during breast cancer metastasis (**Appendix 1**).

6.2 Effects of Platelets on Proteases in Breast Cancer Cells (Int. J. Cancer, 60, 413-417, 1995).

In collaboration with DR. S. Menashi, we investigated how tumor-stroma interactions modulate MMP expression and consequently how they affect tumor cell invasion. It has been shown that interaction of tumor cells with platelets facilitates metastasis of tumor cells. Platelets protect tumor cells against the host's immune defense and enhance tumor-cell extravasation. In the present work we show that platelets increase the invasiveness of breast cancer cell lines (MCF-7, ZR-51 and MDA-MB231) through extracellular matrix, and propose this as an additional mechanism by which platelets facilitate metastasis. Since the gelatinases (MMP-2 and MMP-9) and urokinase have been implicated in degradation of the extracellular matrix and cell migration, and therefore in tumor invasion, we have also analyzed whether the interaction of platelets with tumor cells can modify the secretion of these proteases by tumor cells. MDA-MB231, which was the most invasive cell line among the three tested and was the most potent in inducing platelet aggregation, secreted the highest level of urokinase and was the only one in which gelatinase B (MMP-9) was detected. While platelets had no significant effect on the urokinase activity expressed by these cells, they induced in MDA-MB231 an important increase in the secretion of latent gelatinase B (pro-MMP-9), which can be reproduced by both platelet membrane and platelet releasate of activated platelets. This increase in pro-MMP-9 expression could be responsible, at least in part, for the increased invasiveness of these cells, since added TIMP-1 significantly reduced the number of cells which traversed matrigel (**Appendix 2**).

6.3 Effect of Carbohydrates on pro-MMP-2 Activation in Fibroblasts (Bioch. Biophys. Res. Comm., 228, 530-538, 1996).

These studies were designed to address the mechanism by which concanavalin A induces the activation of pro-MMP-2 in fibroblasts. We investigated the effect of plant lectins, carbohydrates and inhibitors of the cytoskeleton on pro-MMP-2 activation in normal (HLF1) and malignant fibroblast (HT1080) cells. Native ConA induced pro-MMP-2 activation in both cell types while dimeric succinyl-ConA had no effect suggesting that receptor clustering is involved in activation. Wheat germ agglutinin (WGA) also induced proMMP-2 activation. N-acetyl-D-glucosamine (GlcNac) inhibited the effects of ConA and WGA while mannose only inhibited ConA-induced proMMP-2 activation. Mannose also inhibited the expression of MT1-MMP mRNA induced by ConA. Cytochalasin B and colchicine had no effect on the ConA induction of pro-MMP-2 activation. These studies help to define some of the cellular and molecular mechanisms for the induction of proMMP-2 activation (**Appendix 3**).

6.4 Induction of pro-MMP-9 Expression in Breast Cancer Cells by Epidermal Growth Factor (Int. J. Cancer, 70, 722-726, 1997).

In collaboration with Dr. K. Reddy (Dept. of Pathology, Wayne State University) we investigated the effects of epidermal growth factor (EGF), amphiregulin and heregulin on the expression of gelatinases in breast cancer cells. These studies showed that exposure of SKBR-3

cells to EGF or amphiregulin induced pro-MMP-9 expression whereas heregulin had no effect. A tyrosine kinase inhibitor (PD) that inhibited EGF receptor autophosphorylation in response to EGF inhibited EGF-induced cell proliferation but had no effect on pro-MMP-9 expression. This suggests that different signal transduction pathways mediate the effects of EGF on breast cancer cells. Furthermore, these studies also suggest that EGF may contribute to the invasive phenotype by inducing the expression of pro-MMP-9 in EGF receptor-overexpressing breast cancer cells (Appendix 6).

6.5 Surface Association of pro-MMP-9 in Breast Epithelial Cells (Can. Research, 57, 3159-3167, 1997).

Cell surface association of extracellular matrix (ECM)-degrading enzymes has been suggested to facilitate proteolysis of ECM in areas of cell-matrix contacts and to be crucial for the process of tumor cell invasion. Matrix metalloproteinase-9 (MMP-9) is a member of the MMP family of endopeptidases that has been shown to play a critical role in hydrolysis of ECM components and has been localized on the surface of tumor cells. However, the nature of the cell surface association of MMP-9 is unknown. We investigated the cell surface association of pro-MMP-9 in human breast epithelial MCF10A cells treated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Surface biotinylation and immunoprecipitation with anti-MMP-9 antibodies revealed the presence of two MMP-9 forms (92 and 85-kDa) on the surface of TPA-treated MCF10A cells while in the media only the 92-kDa form was detected, mostly in complex with TIMP-1, a specific MMP-9 inhibitor. The MMP-9 forms were also found in purified plasma membranes of TPA-treated cells. In contrast, the plasma membranes contained little or no TIMP-1. The surface-bound MMP-9 forms were recognized by an antibody to the N-terminal prodomain indicating that both represent latent enzymes. Pulse chase analysis and endoglycosidase-H digestion of surface-biotinylated MMP-9 forms demonstrated that the 85-kDa species was endoglycosidase-H sensitive suggesting targeting of the precursor form of MMP-9 to the cell surface. These studies demonstrate a specific cell surface association of MMP-9 in response to TPA that may help to localize TIMP-1 free-enzyme on the surface of breast epithelial cells (Appendix 5).

6.6 Identification of a Pro-MMP-9 Surface Binding Protein in Breast Epithelial Cells (J. Biol. Chem., 273, 10672-10681, 1998).

Association of matrix metalloproteinases (MMPs) with the cell surface and with areas of cell-matrix contacts is critical for extracellular matrix (ECM) degradation. Previously we showed the surface association of pro-MMP-9 in human breast epithelial MCF10A cells. Here, we characterized the binding parameters of pro-MMP-9 and show that the enzyme binds with high affinity ($K_d \sim 22$ nM) to MCF10A cells and other cell lines. Binding of pro-MMP-9 to MCF10A cells does not result in zymogen activation and is not followed by ligand internalization, even after complex formation with tissue inhibitor of metalloproteinase-1 (TIMP-1). A 190-kDa cell surface protein was identified by ligand blot analysis and affinity purification with immobilized pro-MMP-9. Microsequencing and immunoblot analysis revealed that the 190-kDa protein is the $\alpha 2$ (IV) chain of collagen IV. Specific pro-MMP-9 surface binding was competed with purified $\alpha 2$ (IV) and was significantly reduced after treatment of the cells with active MMP-9 before the binding assay since $\alpha 2$ (IV) is hydrolyzed by MMP-9. A pro-MMP-9/TIMP-1 complex and

MMP-9 bind to $\alpha 2(\text{IV})$ suggesting that neither the C-terminal nor the N-terminal domain of the enzyme are directly involved in $\alpha 2(\text{IV})$ binding. The closely related proMMP-2 exhibits a weaker affinity for $\alpha 2(\text{IV})$ compared to that of pro-MMP-9 suggesting that sites other than the gelatin-binding domain may be involved in the binding of $\alpha 2(\text{IV})$ to pro-MMP-9. While proMMP-9 forms a complex with $\alpha 2(\text{IV})$, the proenzyme does not bind to triple helical collagen IV. These studies suggest a unique interaction between pro-MMP-9 and $\alpha 2(\text{IV})$ that may play a role in targeting the zymogen to cell matrix contacts and in the degradation of the collagen IV network (**Appendix 9**).

6.7 Cell Density Regulation of Pro-MMP-2 Binding and Activation in Breast Cancer Cells (Int. J. Cancer, 75, 259-265, 1998).

Degradation of extracellular matrix takes place in areas of cell-matrix contacts and is partly carried out by the action of matrix metalloproteinases (MMP). MMP-2 is a member of the MMP family that has been associated with breast-cancer metastasis. In the present study, we investigated the association of MMP-2 to the surface of breast-cancer cells and revealed an MMP-2-binding site that is expressed on sparsely plated cells and which is progressively lost as the cells approach confluence. Gelatin zymography, immunostaining and flow cytometry of MDA-MB-231 cells from sparse cultures demonstrated binding both of latent and of activated exogenous MMP-2, while little or no binding of MMP-2 was observed in confluent culture. Analysis of the expression of MTI-MMP, TIMP-2 and alpha (v) integrin, three proteins shown to play a role in cell-surface association of MMP-2, revealed enhanced levels of these proteins in confluent MDA-MB-231 cells. Thus, the reduced MMP-2 binding to confluent cells is not related to a deficiency in these MMP-2- binding proteins. Taken together, these studies suggest that MMP-2 binding to the surface of breast-cancer cells is regulated by cell-cell interactions and that tumor cells invading from the main tumor mass can up-regulate their MMP-2-binding capacity to acquire greater invasive capacity (**Appendix 8**).

6.8 Studies from the co-PI, Dr. Bonnie Sloane, partially supported by the Army grant (Cancer Treat. Res., 83, 325-352, 1996; Biochem. J., 318, 793-800, 1996; Med. Asp. Proteases and Inhibitors, N. Katunuma et al. eds., IOS Press, 185-194, 1997).

Studies from the laboratory of Dr. B. Sloane have shown that cathepsin B can be localized on the surface of breast epithelial cells. It is believed that this surface association facilitates degradation of ECM and consequently contributes to cell migration and invasion. Immunofluorescent studies demonstrated that cathepsin B is localized in the periphery of MCF10A cells transformed with the H-ras oncogene. To characterize the mechanism involved in binding of cathepsin B it was necessary to obtain a pure preparation of cathepsin B. In collaboration with Dr. Bonnie Sloane, we expressed human recombinant pro-cathepsin B in the vaccinia virus mammalian cell expression system. The recombinant enzyme was characterized in terms of activity, inhibition and glycosylation. Current studies using the recombinant enzyme are aimed at defining the cathepsin B-binding sites on breast cancer cells. Other current studies are aimed at examining the role of tumor-stroma interactions on the expression of cathepsin B in breast epithelial cells using breast fibroblasts and several breast cancer cell lines. These are ongoing studies and results are still inconclusive (**Appendix 10 and 11**).

6.9 Ongoing Studies on the Activation of Pro-MMP-2 in Breast Fibroblasts.

In our previous report, we informed about our progress on the expression of MT1-MMP and pro-MMP-2 in breast fibroblasts. These studies demonstrated that the breast fibroblasts can be induced to activate pro-MMP-2 in response to a collagen I matrix. In addition, we showed the processing of MT1-MMP in these cells by pulse-chase analysis and the effects of collagen I and ConA on the levels of MT1-MMP mRNA by northern blot analysis. We could not find a modulation of pro-MMP-2 activation and/or any effect on TIMP-2 or MT1-MMP expression in the breast fibroblasts in response to breast cancer cells using co-cultures or conditioned media. Therefore, our original hypothesis that interactions of stromal cells with breast cancer cells modulate pro-MMP-2 expression and activation could not be verified in these *in vitro* studies. In the last six month we have been investigating how collagen I induces pro-MMP-2 activation and MT1-MMP processing. The purpose of these studies is to unveil the cellular mechanism that induces pro-MMP-2 activation. These studies are still in progress.

7. CONCLUSIONS

From published observations

1. We have described a novel mechanism of pro-MMP-9 activation involving MMP-2 at the cell surface. This process may explain in part how enzymes produced by tumor stromal cells are activated on the surface of breast cancer cells.
2. Metastatic MDA-MB-231 breast cancer cells express pro-MMP-9 in response to platelets resulting in enhanced *in vitro* invasion. Induction of pro-MMP-9 in circulating metastatic breast cancer cells by platelets may contribute to tumor cell intravasation and formation of distant metastasis.
3. Pro-MMP-9 associates with the surface of breast epithelial cells after treatment with phorbol ester. Surface-bound pro-MMP-9 appears to be free of TIMP-1 in spite of the high levels of inhibitor in the supernatant. This suggests that surface association may preclude formation of enzyme/inhibitor complexes a process that may have significant consequences for enzyme activation and activity.
4. EGF induces expression of pro-MMP-9 in breast cancer cells in a process that is independent from its mitogenic activity. Thus, EGF may play a role in promoting invasive behavior of breast cancer cells.
5. We have identified the $\alpha 2(\text{IV})$ chain of type IV collagen as a major binding site for pro-MMP-9 on the surface of breast epithelial cells. Binding of pro-MMP-9 to surface-bound $\alpha 2(\text{IV})$ may help to retain the enzyme at areas of cell-matrix contacts. These studies have also identified the chain involved in binding pro-MMP-9 to collagen IV, a major substrate of this enzyme and a known barrier for tumor cell invasion.
6. Binding of pro-MMP-2 to the surface of MDA-MB-231 breast cancer cells is dependent on cell density and is independent on the levels of expression of MT1-MMP, TIMP-2 or the

integrin $\alpha v \beta 3$. These studies suggest that additional factors are likely to play a role in the association of pro-MMP-2 to the surface of breast cancer cells.

7. Cathepsin B is expressed by breast cancer cell lines and it can be found on the cell surface. The enzyme is localized in the perinuclear region and in cytoplasmic projections on the basolateral region of the cells.

From ongoing studies.

8. We have found induction of proMMP-2 activation in primary breast fibroblasts grown on collagen I gels. In addition, both cathepsin B and D protein levels were slightly increased on breast fibroblasts grown on collagen I.
9. Pulse chase analysis demonstrated no significant differences in the processing of MT1-MMP on breast fibroblasts grown on collagen I or on plastic. However, northern blot analysis demonstrated a 2-fold induction of MT1-MMP mRNA in primary breast fibroblasts cultured on collagen I.

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11. PERSONNEL

Rafael Fridman
Marie Dehem
David Gervasi
Karen Kernacki
Priya Menon
Daniel Pena
Marta Toth
Adam Treitman
Grace Ziegler
Yuval Raz
Daniel Visscher

Activation of Progelatinase B (MMP-9) by Gelatinase A (MMP-2)¹

Rafael Fridman,² Marta Toth, Daniel Peña, and Shahriar Mobashery

Departments of Pathology [R. F., M. T., D. P.] and Chemistry [S. M.], Wayne State University, Detroit, Michigan 48201

ABSTRACT

The M_r 72,000 (MMP-2; gelatinase A) and M_r 92,000 (MMP-9; gelatinase B) gelatinases are two members of the family of matrix metalloproteinases (MMPs). These proteinases are thought to play a critical role in tumor cell invasion and are frequently coexpressed in human cancers. Gelatinases are secreted in a latent inactive form, and their conversion to the active species can be accomplished by other proteolytic enzymes, including other MMPs. We report herein that organomercurial or plasma membrane-activated M_r 72,000 gelatinase A activates progelatinase B to an M_r 82,000 active form in a process inhibited by tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2. Progelatinase B activation was accomplished by the two active species of gelatinase A, the M_r 62,000 and M_r 45,000 forms, generated after plasma membrane or organomercurial activation of TIMP-2-free progelatinase A. The M_r 45,000 species of gelatinase A lacks both the NH_2 -terminal profragment and the $COOH$ -terminal domain known to play a role in plasma membrane activation and the regulation of TIMP-2 inhibition. These results suggest a novel mechanism of activation of progelatinase B mediated by gelatinase A species that may be localized in the surface of tumor cells and enhance matrix degradation during cancer metastasis.

INTRODUCTION

Tumor metastasis requires proteolytic degradation of ECM³ components to facilitate the invasion of basement membranes and connective tissue matrices by the malignant cancer cells. Several groups of proteases have been implicated in tumor cell invasion including MMPs (1, 2), serine proteases (3), and cysteine proteases (4). The M_r 72,000 gelatinase A/type IV collagenase (MMP-2; Ref. 5) and the M_r 92,000 gelatinase B/type IV collagenase (MMP-9; Ref. 6) are two members of the MMP family postulated to play a critical role in tumor invasion and angiogenesis (1, 7). Elevated levels of these enzymes were reported in human cancers (7-14), and the metastatic potential of tumor cells in experimental models of metastasis has been correlated with the expression and activity of these proteinases (reviewed in Ref. 7). The association of the gelatinases with malignancy may be related to their ability to degrade basement membrane collagen IV to yield 1/4 NH_2 -terminal and 3/4 $COOH$ -terminal fragments (5, 6, 15). For that reason, these proteinases are also known as type IV collagenases (5, 6). In addition, they degrade gelatin and thus are named gelatinases (2, 16). Gelatinases degrade other ECM components *in vitro*, including collagens V, VIIs and XI; fibronectin; laminin (2, 5, 6, 16); elastin (17, 18); proteoglycans (18-20); and entactin (21), although with different efficiencies and probably involving different cleavage sites. They may also attack other biologically relevant molecules. For example, gelatinase A hydrolyzes the Lys¹⁶-Leu¹⁷ peptide bond of a synthetic decapeptide representing the soluble β -amyloid sequence of amino-acid residues 10-20 (22) and the Lys¹⁶-Leu¹⁷ and Met³⁵-Val³⁶

peptide bonds of β -amyloid peptides 1-40 and 1-42, respectively, isolated from brains of Alzheimer's disease patients (23). We have shown recently that the gelatinases can hydrolyze galectin-3, a cell surface lectin involved in cell-cell and cell-matrix interactions and metastasis (24). Thus, the spectrum of proteins that can be potentially cleaved by these MMPs suggests an important role for these proteinases in the regulation of various biological processes.

The progelatinases, like other members of the MMP family, are secreted in a latent form that requires activation (1, 2, 16). Thus, proenzyme activation is a critical event in the regulation of gelatinase activity and may be essential for ECM degradation during tumor cell invasion (1, 7). The physiological mechanisms responsible for activation of the progelatinases are not completely understood but may involve the action of other proteases, including other MMPs. Previous studies (25-27) have shown a plasma membrane-dependent activation specific for progelatinase A, possibly mediated by a recently identified MT-MMP (28). The plasma membrane-dependent activation of progelatinase A is induced in cultured cells by treatment with phorbol ester (26, 27), concanavalin A (25, 26, 29, 30), transforming growth factor β (26), or a collagen substrate (31). Plasma membrane activation of progelatinase A was shown to generate the reported M_r 62,000 active species with the NH_2 -terminal sequence starting at Tyr⁸¹ but also to generate an additional active species of M_r 41,000-45,000 (27) with high specific activity (29, 30). A similar pattern of activation was observed after organomercurial activation of progelatinase A free of TIMP-2 (32, 33), a specific inhibitor of the M_r 72,000 enzyme known to form a noncovalent complex with the proenzyme form (34, 35). We (32) have reported previously that the M_r 45,000 species showed an electrophoretic mobility similar to a recombinant truncated gelatinase A lacking the $COOH$ -terminal domain, suggesting that formation of the M_r 45,000 form also involved a cleavage at the $COOH$ -terminal region. Interestingly, the $COOH$ -terminal domain of progelatinase A is the TIMP-2 binding domain (32, 36-38) in the proenzyme form. Removal of this domain does not impair catalytic activity but reduces the rate of TIMP-2 inhibition (32, 37, 38). Thus, the nature of the active species of gelatinase A, formed after plasma membrane activation, may regulate enzyme activity and inhibition by TIMP-2.

Progelatinase B, in contrast to the M_r 72,000 enzyme, appears not to be activated by a plasma membrane-dependent mechanism and is usually detected in the culture media of normal and tumor cells in a latent form (25-27). Studies with purified enzymes, however, have shown the ability of several proteases to activate progelatinase B. These include stromelysin-1 (MMP-3; Refs. 39 and 40), plasmin (41), and tissue kallikrein (42). Activation of progelatinase B with stromelysin-1, which is most efficient (40), generates an M_r 82,000 active species with enzymatic activity (39, 40). The coordinated regulation of progelatinase B and stromelysin-1 expression by cytokines in certain cells has been suggested to facilitate progelatinase B activation (39). However, in some tumors, these enzymes are not always coexpressed, in contrast to gelatinase A and B. For example, high levels of gelatinase A and B were detected in breast tumors (43-45), whereas stromelysin-1 mRNA was undetectable (43). We have also reported enhanced expression of both gelatinases in breast tumors by immunohistochemistry (46). High levels of both gelatinases were also reported in colon (10) and bladder (14) cancers. Due to the frequent

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² To whom requests for reprints should be addressed, at Department of Pathology, School of Medicine, Wayne State University, 540 East Canfield Avenue, Detroit, MI 48201.

³ The abbreviations used are: ECM, extracellular matrix; MMP, matrix metalloproteinase; MT-MMP, membrane-type MMP; TIMP, tissue inhibitor of metalloproteinase; APMA, *p*-aminophenylmercuric acetate.

coexpression of the M_r 72,000 and M_r 92,000 gelatinases in human tumors, we chose to investigate the ability of these enzymes to activate each other. Here we show that gelatinase A can activate progelatinase B to generate an M_r 82,000 active form. This process was observed after plasma membrane activation of progelatinase A and could be mediated by either of the active species of gelatinase A. In addition, progelatinase B activation by gelatinase A was inhibited by TIMP-1 and TIMP-2. These results suggest a novel, but not exclusive, mechanism for progelatinase B activation that may enhance ECM degradation in certain tumors and possibly contribute to tumor cell invasion.

MATERIALS AND METHODS

Expression and Purification of Recombinant Enzymes and Inhibitors. Human recombinant progelatinase A, progelatinase B, TIMP-2, and TIMP-1 were all expressed in mammalian cells using a recombinant vaccinia virus expression system (Vac/T7), as described previously (32, 47). The cDNA for human progelatinase B was a generous gift from Drs. K. Tryggvason and A. Tuuttila (University of Oulu, Oulu, Finland). TIMP-1 cDNA was kindly provided by Dr. Stetler-Stevenson (NIH, Bethesda, MD). Recombinant vaccinia viruses containing either the progelatinases or TIMP cDNAs were obtained by homologous recombination as described previously (32, 47).

Pregelatinase A and B were purified from the media (Opti-MEM I; GIBCO, Grand Island, NY) of HeLa cells infected with the appropriate recombinant viruses, as described previously (32, 33). TIMP-2 was purified by affinity chromatography using a mAb (CA-101) against human TIMP-2, as described previously (32, 33). TIMP-1 was purified from the media of infected HeLa cells using a lentil lectin-Sepharose 4B (Sigma Chemical Co.); the column was equilibrated with 20 mM HEPES (pH 7.5), 500 mM NaCl, 1 mM CaCl_2 , 10% glycerol, 0.05% Brij-35, and 0.02% NaN_3 (48). TIMP-1 was eluted with 500 mM methyl α -D-mannopyranoside (Sigma) diluted in the same buffer, dialyzed against 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 5 mM CaCl_2 , and 0.02% Brij-35 (collagenase buffer), concentrated in a Centricon-10 concentrator (Amicon, Beverly, MA), and purified by reverse phase-HPLC (48). The concentrations of the purified enzymes and TIMPs were determined by amino acid analysis (49).

Purification of Gelatinase A Active Species. To isolate the active forms of gelatinase A, 1–2 mg of progelatinase A in 20 mM Tris/HCl (pH 7.5), 5 mM CaCl_2 , and 0.02% Brij-35 were activated with 1 mM APMA for 1 h at 37°C. The activated gelatinase A was applied to a red-agarose (Sigma) column (10 x 0.5 cm) equilibrated with the same buffer. A gradient of 0.05–3 M NaCl in equilibrating buffer was then applied to the column. The M_r 45,000 species was recovered in the void volume, whereas the M_r 62,000 species eluted with 3 M NaCl. To separate other activation fragments from the M_r 62,000 species, the sample was dialyzed against collagenase buffer and was subjected to gelatin affinity chromatography. Three fragments of M_r 32,000, 26,000, and 12,000 were recovered in the flow through, whereas the M_r 62,000 species was eluted from the gelatin column with 10% DMSO in the collagenase buffer. The purified M_r 62,000 and M_r 45,000 species were concentrated with a Centricon-10 concentrator and analyzed by zymography and SDS-PAGE. Protein concentrations of the purified active species were determined by amino acid analysis (49).

Microsequence Analysis. The activation species of either gelatinase A or gelatinase B were separated by SDS-PAGE under reducing conditions and transferred to an Immobilon membrane (Millipore, Marlboro, MA). The transferred proteins were stained with 1% amido black in 20% isopropanol and 10% acetic acid, and the appropriate stained bands were cut out of the membrane. The NH_2 -terminal sequence of the immobilized proteins was determined on an Applied Biosystems 475A gas-phase protein sequencer.

Enzyme Assays. Zymography was performed in 10% SDS-polyacrylamide gels containing 0.1% gelatin using precast mini-gels from Novex (Encinitas, CA), as described earlier (32, 33). Samples of the purified enzymes were resuspended in the Laemmli sample buffer without reducing agents and were not subsequently heated. Gelatinase activity was determined using heat-denatured rat $[^3\text{H}]$ collagen type I (18,000 cpm/ μg ; Dupont NEN, Wilmington, DE) as the substrate (~60,000 cpm/reaction) as described previously (32, 33).

Immunoblots. Purified samples were subjected to SDS-PAGE under reducing conditions. The separated proteins were transferred to nitrocellulose paper. After blocking with 3% BSA and 3% nonfat dry milk in 50 mM Tris/HCl (pH 7.5), the blots were incubated with the corresponding primary antibody diluted in 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween 20. The immunodetection of the antigen was performed using the immunoperoxidase ABC kit (Vector Laboratories). The characterization of the mAbs to the gelatinases and TIMP-2 was described previously (46, 50). A rabbit polyclonal antibody against a synthetic peptide comprising residues 513 to 530 of human progelatinase A was a generous gift from Dr. Steven Ledbetter (Upjohn Co., Kalamazoo, MI).

Activation of Pregelatinases. Pregelatinase A was activated with either APMA or plasma membrane. For APMA activation, progelatinase A in collagenase buffer was incubated (30 min at 37°C) with 1 mM APMA (final concentration) prepared from a 10X APMA solution in 50 mM NaOH. To remove APMA, the active gelatinase A was applied to a Quick-Spin column (Boehringer Mannheim, Indianapolis, IN) that was equilibrated previously with collagenase buffer. The concentration of active gelatinase A was then determined by titration with a known amount of TIMP-2, assuming a 1:1 stoichiometry for complete inhibition (32, 33, 51). Plasma membrane activation of progelatinase A (27) was performed using plasma membranes isolated from HT1080 cells treated with phorbol ester (a generous gift from Dr. Greg Goldberg, Washington University, St. Louis, MO). Briefly, progelatinase A (50–200 ng) diluted in 25 mM Hepes/KOH (pH 7.5) and 0.1 mM CaCl_2 was incubated (1–3 h) with 10 μg of isolated plasma membrane, as described previously (27). The presence of active gelatinase A species was determined by zymography or immunoblot analysis.

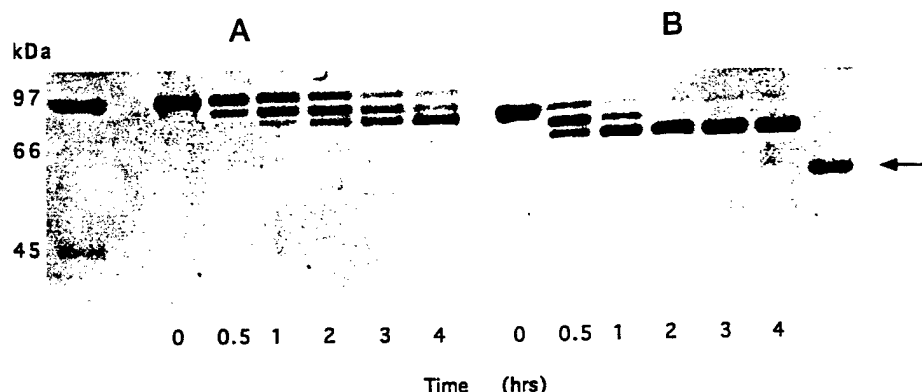
To activate progelatinase B with gelatinase A, progelatinase B was diluted in collagenase buffer, and each solution was individually incubated (37°C for various periods of time) with either activated gelatinase A (by APMA or plasma membranes) or purified M_r 62,000 or M_r 45,000 species. The reaction was then stopped by the addition of the Laemmli sample buffer containing 1% β -mercaptoethanol. Conversion to the active forms was determined after separation of the samples by SDS-PAGE and staining of the gels with 0.25% Coomassie Brilliant Blue R-250 in 10% methanol/5% acetic acid or by immunoblot analysis using a mAb (CA-209) to human progelatinase B. In some experiments, progelatinase B was incubated with stoichiometric amounts of TIMP-1 or TIMP-2 for 20 min at 22°C before activation with gelatinase A. Pregelatinase B was also activated in concentrated conditioned media of HT1080 cells by adding various concentrations of exogenous recombinant gelatinase A species. To obtain conditioned medium, a confluent T75 tissue culture flask was incubated (16 h at 37°C) with 7 ml of serum-free DMEM in a CO_2 incubator. The medium was then collected, clarified by centrifugation (10 min at 2000 rpm), and concentrated (10-fold) using a Centricon-10 concentrator.

RESULTS

Activation of Pregelatinase B by Gelatinase A. We examined the ability of gelatinase A to activate progelatinase B. Incubation of progelatinase B with APMA-activated gelatinase A resulted in a time- and dose-dependent conversion of the M_r 92,000 zymogen to the M_r 82,000 species (Fig. 1). An intermediary form of M_r 86,000 could be observed as early as 30 min after the addition of gelatinase A. Full conversion to the M_r 82,000 species was observed after a 2-h incubation period (Fig. 1B; 1:50 gelatinase A:progelatinase B molar ratio). To rule out the possibility that trace amounts of APMA remaining in the gelatinase A preparation were responsible for the activation of progelatinase B, the M_r 92,000 zymogen was incubated with a 10-fold excess APMA (10 mM) for 1 h. Under these conditions, APMA failed to fully convert progelatinase B to the M_r 82,000 form (data not shown). Gelatinase B did not activate progelatinase A (data not shown).

The NH_2 -terminal sequences of the M_r 86,000 and the M_r 82,000 gelatinase B species formed after activation by gelatinase A were determined and found to be Met⁴¹ for the M_r 86,000 form and Phe⁸⁸ for the M_r 82,000 form. These sequences are the same as

Fig. 1. SDS-PAGE analysis of the activation of progelatinase B by gelatinase A. Human recombinant progelatinase A was activated with 1 mM APMA (1 h at 37°C) and applied to a Quick Spin column to remove the APMA as described in "Materials and Methods." The activated gelatinase A was incubated (37°C) with human recombinant progelatinase B for various time periods at a 1:100 (A) or 1:50 (B) gelatinase A:progelatinase B molar ratio. At the end of the indicated incubation times, the samples (1 µg/lane of progelatinase B) were analyzed in 10% SDS-polyacrylamide gel under reducing conditions, followed by staining of the gel with 0.25% Coomassie Brilliant Blue. *Left lane*, molecular weight standards (Bio-Rad). *Arrow on the right*, the activated gelatinase A used in the experiment.



those reported previously for the activation of progelatinase B by stromelysin-1 (39).

Plasma Membrane-activated Gelatinase A Activates Progelatinase B. Progelatinase A can be activated by APMA (5) or by plasma membrane containing MT-MMP (25–28). We reported previously that APMA activation of TIMP-2-free progelatinase A generated two active species of M_r 62,000 and M_r 45,000 (32, 33). We compared the pattern of activation of progelatinase A by APMA and plasma membrane of HT1080 cells treated with phorbol ester. As shown in Fig. 2,

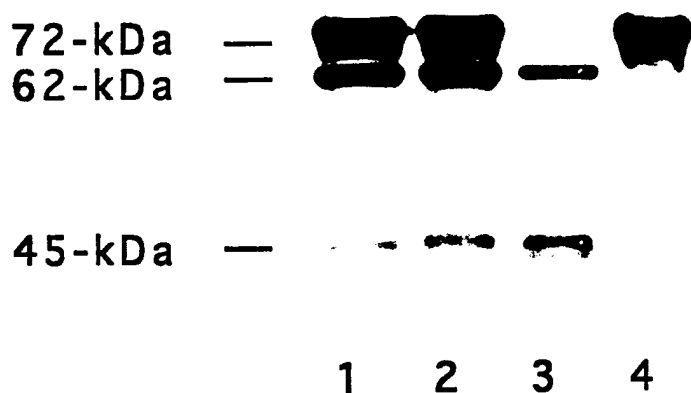


Fig. 2. Activation of progelatinase A by plasma membranes or APMA. Progelatinase A (200 ng/lane) in 25 mM HEPES/KOH (pH 7.5)-0.1 mM CaCl_2 was incubated (37°C) with either 10 µg of HT1080 plasma membranes (Lane 1, 1 h; Lane 2, 2 h) or with 1 mM APMA for 1 h (Lane 3) or with buffer alone (Lane 4). The samples were then separated by electrophoresis in a 10% SDS-polyacrylamide gel under reducing conditions, followed by transfer to nitrocellulose paper. The blot was developed using a mAb to progelatinase A as described in "Materials and Methods." Molecular weights on the left indicate the relative mass of the gelatinase A species.

plasma membrane- (Fig. 2, Lanes 1 and 2) or APMA- (Fig. 2, Lane 3) activated progelatinase A into a M_r 62,000 and a M_r 45,000 species. An intermediary form of M_r 64,000 to 66,000 could also be observed (not visible in Fig. 2). Thus, both APMA and plasma membrane activation of progelatinase A generate the M_r 62,000 and M_r 45,000 species.

Since APMA activated-gelatinase A caused activation of progelatinase B, we wished to examine the effect of plasma membrane-activated gelatinase A on progelatinase B activation. To this end, the plasma membranes from phorbol ester treated-HT1080 cells were incubated (3 h at 37°C) with progelatinase A, and then progelatinase B was added to the reaction mixture for an additional 3-h incubation period. The reaction mixture was then analyzed by immunoblot using a mAb (CA-209) against progelatinase B and a mAb (CA-801) against progelatinase A. These studies (Fig. 3) showed that plasma membrane activated-gelatinase A caused the conversion of progelatinase B to the M_r 86,000 and M_r 82,000 species (Fig. 3, Lanes 1 and 2). The immunoblot also showed the bands corresponding to the plasma membrane-activated gelatinase A, including a M_r 64,000, 62,000, and a 45,000 form. When progelatinase A was incubated (3 h at 37°C) with plasma membranes in the presence of recombinant TIMP-2, there was a significant reduction in the formation of the M_r 62,000 and M_r 45,000 species, and most of the M_r 72,000 enzyme remained in the latent form (Fig. 3, Lanes 3 and 4). Consistently, the addition of progelatinase B to the mixture of plasma membranes, progelatinase A, and TIMP-2, followed by another 3-h incubation period, had no effect on the activation of progelatinase B (Fig. 3, Lanes 3 and 4). Also, progelatinase B incubated with plasma membranes in the absence of progelatinase A was not activated (data not shown), as reported previously (25–27). Taken together, these studies demonstrate that

Fig. 3. Progelatinase B activation by plasma membrane-activated gelatinase A and inhibition by TIMP-2. Plasma membranes (10 µg/reaction) were incubated (3 h at 37°C) with progelatinase A (200 ng/reaction) in 25 mM HEPES/KOH (pH 7.5)-0.1 mM CaCl_2 in the absence (Lanes 1 and 2) or presence (Lanes 3 and 4) of stoichiometric amounts (60 ng/reaction) of TIMP-2 to progelatinase A. An aliquot of the reaction mixture was then incubated with progelatinase B (200 ng/lane) at 1:10 (Lanes 1 and 3) and 1:50 (lanes 2 and 4) molar ratios (gelatinase A:progelatinase B) for another 3 h at 37°C. The mixtures were then separated by electrophoresis in a 10% SDS-polyacrylamide gel under reducing conditions, followed by transfer to nitrocellulose paper. The blot was developed using a mixture of mAbs to progelatinase A and B, as described in "Materials and Methods." Molecular weights on the left represent the prestained molecular weight standards (low range; Bio-Rad).

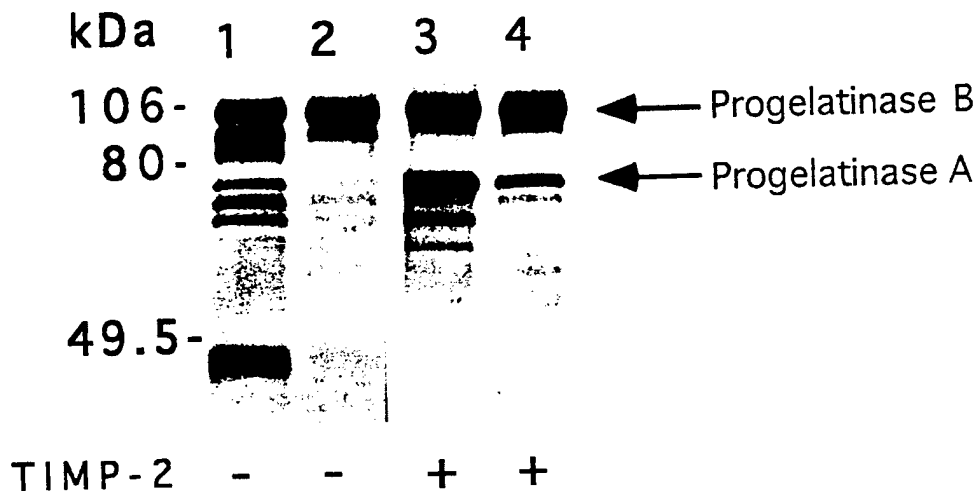
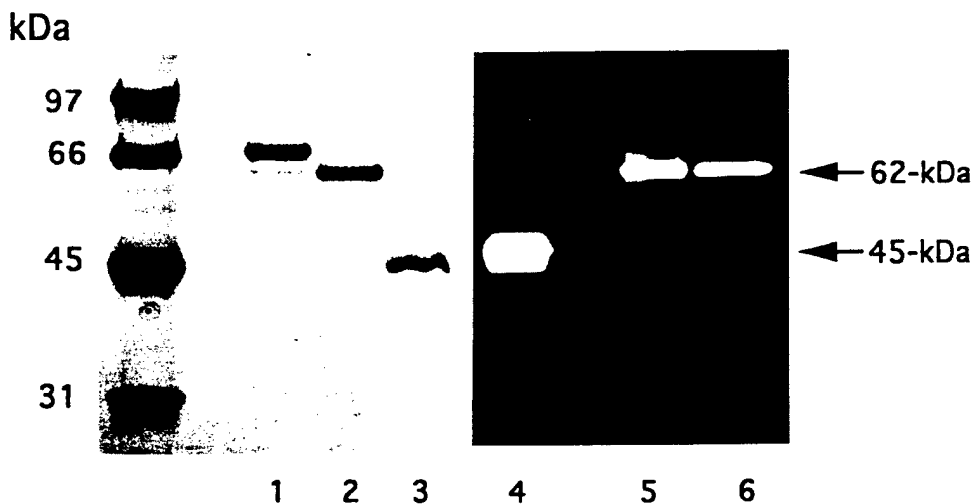


Fig. 4. Purification of the M_r 62,000 and M_r 45,000 species of gelatinase A. The active M_r 62,000 and M_r 45,000 species were purified as described in "Materials and Methods" and analyzed by SDS-PAGE (Lanes 1–3) under reducing conditions and by gelatin-zymography (Lanes 4–6). Lane 1, progelatinase A (1 μ g); Lane 2, purified M_r 62,000 species (1 μ g); Lanes 3 and 4, purified M_r 45,000 species, 1 μ g and 1 ng, respectively; Lane 5, low salt (0.5 M NaCl) fraction (10 μ l) from the red agarose column showing elution of the M_r 62,000 species with some M_r 45,000 form; and Lane 6, high salt (3 M NaCl) fraction (10 μ l) containing the M_r 62,000 species. The left lane shows the molecular weight standards (low range; Bio-Rad).



both APMA and plasma membrane-activated gelatinase A activate progelatinase B.

Isolation of Gelatinase A Species. To demonstrate the ability of the gelatinase A species to activate progelatinase B, it was necessary to isolate the M_r 62,000 and the M_r 45,000 forms. To this end, progelatinase A was activated with APMA, and the active species were separated by red-agarose chromatography using a NaCl gradient. SDS-PAGE analysis and zymography of the purified M_r 45,000 species, which eluted in the void volume, revealed a single active band of M_r 45,000 (Fig. 4, Lanes 3 and 4). Increasing concentrations of NaCl resulted in a stepwise elution of the M_r 62,000 active species (Fig. 4, Lane 5, 0.5 M NaCl; both the M_r 45,000 and the M_r 62,000 forms were observed). At high salt concentrations (3 M NaCl), a single active form of M_r 62,000 was detected (Fig. 4, Lane 6). The high-salt fraction containing the M_r 62,000 enzyme also showed three additional inactive fragments of M_r 32,000, 26,000, and 12,000 that separated as three distinct bands in SDS-PAGE under reducing conditions (data not shown). To remove these fragments from the M_r 62,000 active species, the dialyzed sample was applied to a gelatin agarose column, and a single protein of M_r 62,000 kDa was eluted with DMSO (Fig. 4, Lane 2); the three smaller fragments did not bind to the gelatin column. NH_2 -terminal sequence of the M_r 62,000 and the M_r 45,000 species revealed the same NH_2 terminus, which started from Tyr⁸¹. The M_r 32,000 fragment revealed a major and a minor

component. The NH_2 terminus of the former started from Tyr⁴¹⁶ (YGASPDIDLG) and that of the latter from Gly⁴¹⁷ (GASPDIDLG). The NH_2 terminus of the M_r 26,000 fragment also started from Tyr⁴¹⁶. Thus, the M_r 32,000 and 26,000 fragments may represent the COOH-terminal domain of progelatinase A.

Immunoblot analysis using a polyclonal antibody directed against the COOH-terminal end (residues 513–530) of progelatinase A showed that the antibody failed to react with the purified M_r 45,000 species (Fig. 5, Lane 6), consistent with the deletion of the COOH-terminal domain in this species. In contrast, this antibody reacted with the proenzyme form and the M_r 62,000 species of activated gelatinase A (Fig. 5, Lanes 4 and 5, respectively). As expected, a mAb (CA-801) to human progelatinase A, known to bind to the COOH-terminally truncated recombinant M_r 72,000 enzymes (32), reacted with the three enzymes forms (Fig. 5, Lane 1, progelatinase A; Lane 2, activated gelatinase A; and Lane 3, M_r 45,000 species). Similar results were obtained when plasma membrane-activated M_r 72,000 enzyme was used as antigen (data not shown).

Activation of Progelatinase B by the Active Species of Gelatinase A. The purified active species of gelatinase A were used to evaluate their ability to activate progelatinase B. Fig. 6 shows that the purified M_r 45,000 species activated progelatinase B (Fig. 6) to the M_r 86,000 and 82,000 forms in a time- and dose-dependent manner. A similar pattern of activation was obtained when purified M_r 62,000 species was used as activator of progelatinase B (results not shown). Thus, both gelatinase A species activate progelatinase B.

We wished to examine the ability of the gelatinase A species to activate the progelatinase B present in the conditioned media of human fibrosarcoma HT1080 cells. A 100- μ l portion of the 10-fold concentrated conditioned media were incubated (1 h at 37°C) with 0.1 and 1 μ g of purified M_r 45,000 species. The medium was then analyzed for M_r 92,000 enzyme activation by immunoblot using a mAb against human progelatinase B (46). As shown in Fig. 7 (Lanes 3 and 4), the M_r 45,000 species activated the endogenous progelatinase B to the M_r 86,000 and M_r 82,000 species as shown with recombinant progelatinase B. Conditioned medium incubated under the same conditions, but without gelatinase A species, showed no evidence of spontaneous progelatinase B activation (Fig. 7, Lane 2). This experiment suggests that natural progelatinase B can be activated by the gelatinase A active species.

Gelatinase Activity of Gelatinase B and Effects of TIMPs. We measured the activity of gelatinase B against soluble [³H]gelatin after treatment with the M_r 45,000 species. As shown in Fig. 8, incubation of progelatinase B with the M_r 45,000 species generated an enzyme

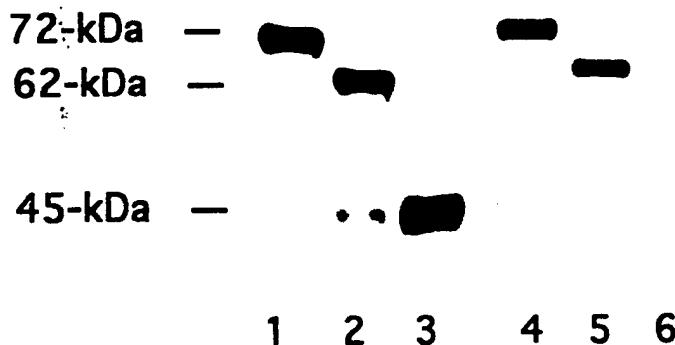
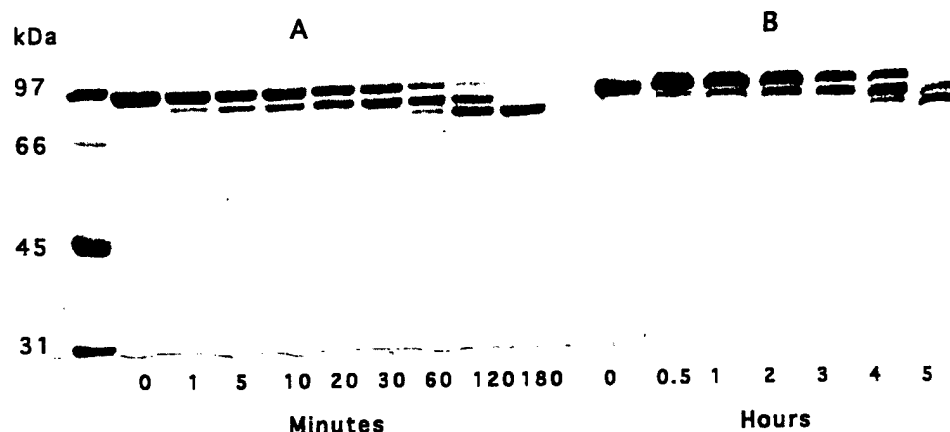


Fig. 5. Immunoblot analysis of activated gelatinase A and the M_r 45,000 species. Samples (200 ng/lane) of progelatinase A (Lanes 1 and 4), APMA-activated gelatinase A (Lanes 2 and 5) and purified M_r 45,000 species (Lanes 3 and 6) were subjected to SDS-PAGE under reducing conditions, followed by blotting to nitrocellulose paper. After blocking, the blots were incubated with a mouse mAb (CA-801) against human progelatinase A (Lanes 1–3) or with a rabbit polyclonal antibody against a synthetic peptide comprising residues 513–530 of human progelatinase A (Lanes 4–6). Molecular weights of the left indicate the relative mass of the purified proteins.

Fig. 6. SDS-PAGE analysis of the activation of progelatinase B by the M_r 45,000 species of gelatinase A. Samples of purified progelatinase B (1 μ g/lane) were incubated (37°C) with purified M_r 45,000 species at 1:10 (A) or 1:100 (B) molar ratios of M_r 45,000:progelatinase B for various periods of time and then subjected to SDS-PAGE analysis under reducing conditions and staining of the gels with 0.025% Coomassie blue. Left lane, the molecular weight standards (low range; Bio-Rad).



with gelatinase activity. The M_r 45,000 species alone also showed activity against [3 H]gelatin that was 3-fold lower than the activity of the gelatinase B/ M_r 45,000 species. In contrast, progelatinase B showed virtually no enzymatic activity.

The effect of TIMP-1 and TIMP-2 on the activation of progelatinase B by gelatinase A species was examined by measuring the activity against [3 H]gelatin in the presence or absence of inhibitor (Table 1). To this end, progelatinase B was incubated with stoichiometric amounts of either TIMP-1 or TIMP-2 before the addition of the gelatinase A species. As controls, we measured the activity of progelatinase B (0.1 μ M) and the M_r 45,000 species alone (0.01 μ M). As shown in Table 1, the purified M_r 45,000 species alone showed a significantly higher (27% more) activity than progelatinase B, as expected. Incubation of progelatinase B with one-tenth molar concentrations of the M_r 45,000 species generated high enzymatic activity (considered 100%). When progelatinase B was incubated with either TIMP-1 or TIMP-2 prior to the addition of the M_r 45,000 species, the presence of the inhibitors caused a complete inhibition of enzymatic

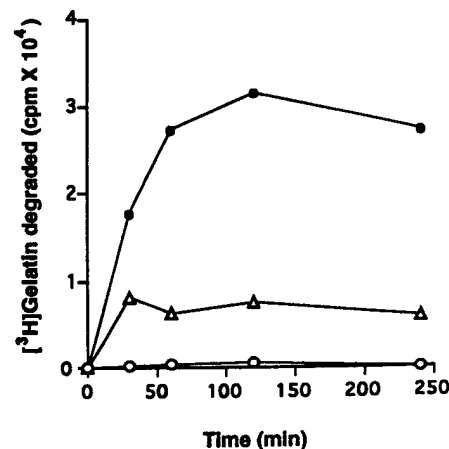


Fig. 8. Enzymatic activity of gelatinase B activated by the M_r 45,000 species. Progelatinase B (0.1 μ M) was incubated with (●) or without (○) the M_r 45,000 species at a 1:10 molar ratio of M_r 45,000:progelatinase B for various time periods and analyzed for activity against soluble [3 H]gelatin, as described in "Materials and Methods." As control, samples of the M_r 45,000 species (0.01 μ M) were incubated alone (Δ) and tested for activity. Soluble degraded [3 H]gelatin was quantified by scintillation spectrometry. Points, duplicate samples that varied by less than 15% (cpm) from each other. Similar results were obtained in three independent experiments.

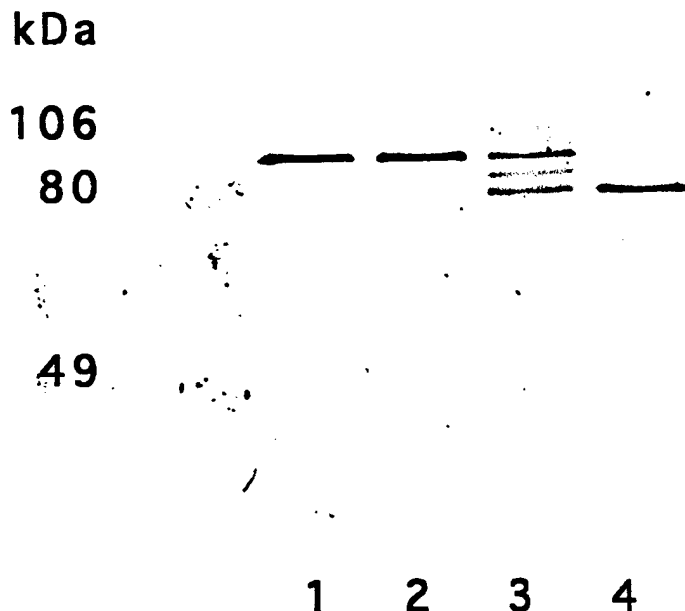


Fig. 7. Activation of progelatinase B in the conditioned media of HT1080 cells by the M_r 45,000 species of gelatinase A. Concentrated (100 μ l/lane) serum-free conditioned medium of HT1080 cells prepared as described in "Materials and Methods" was incubated (1 h at 37°C) with either 0.1 (Lane 3) or 1 μ g of purified M_r 45,000 species or alone (Lane 2). The samples were then subjected to SDS-PAGE under reducing conditions and immunoblot analysis using a mAb (CA-209) against human progelatinase B. Lane 1, the conditioned media not incubated at 37°C. Left lane, the molecular weight of prestained standards (low range; Bio-Rad).

Table 1 Effect of TIMP-1 and TIMP-2 on the activation of progelatinase B by the M_r 45,000 species

Pregelatinase B (0.1 μ M) was incubated (20 min at 22°C) in the presence (+) or absence (–) of equimolar concentrations of either TIMP-1 or TIMP-2 and then incubated (2 h at 37°C) in the presence or absence of the M_r 45,000 species (0.01 μ M). The mixtures were then tested for activity against soluble [3 H]gelatin, as described in "Materials and Methods." Data are expressed as the percentage of activity (solubilized counts) remaining relative to the activity of M_r 45,000-activated gelatinase B (100%).

Enzyme	TIMP-1	TIMP-2	% activity remaining
Pregelatinase B	–	–	1
M_r 45,000	–	–	27
Pregelatinase B/ M_r 45,000	–	–	100
Pregelatinase B/ M_r 45,000	+	–	1
Pregelatinase B/ M_r 45,000	–	+	0

activity. Similar results were obtained when progelatinase B was incubated with or without TIMPs with the purified M_r 62,000 species.

DISCUSSION

In this study, we show that gelatinase A can activate progelatinase B to a M_r 82,000 form with activity against gelatin. Activation of progelatinase B was observed with both plasma membrane or APMA-activated, M_r 72,000 enzyme and with both recombinant and natural progelatinase B. Activation of progelatinase B by gelatinase A is

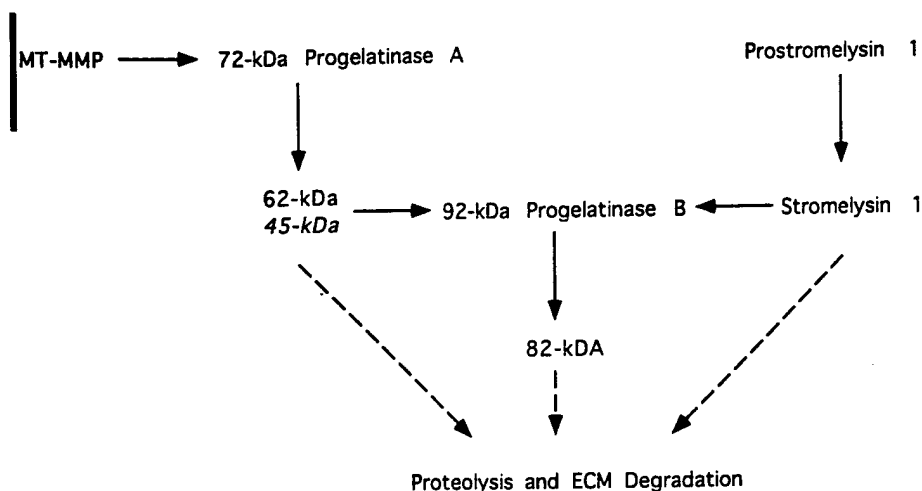


Fig. 9. Schematic model of progelatinase B activation.

accomplished by cleavage at the Glu⁴⁰-Met⁴¹ amide bond to generate a M_r 86,000 form and cleavage at the Arg⁸⁷-Phe⁸⁸ amide bond to generate a M_r 82,000 species in a process similar to the activation of progelatinase B by stromelysin-1 (39). The formation of these species is probably due to the action of gelatinase A alone and does not involve an autocatalytic process, since conversion of the M_r 86,000 species to the M_r 82,000 form was not observed without stromelysin-1 (39). The activation of progelatinase B by gelatinase A observed here is in contrast to the findings of Ogata *et al.* (39), who did not observe any activation of progelatinase B by gelatinase A. This discrepancy may be due to the source of the M_r 72,000 enzyme used for activation of progelatinase B in experiments by Ogata *et al.* (39). Progelatinase A isolated from HT1080 cell culture media [as described in the study of Ogata *et al.* (39)] is expected to be in complex with TIMP-2 (34, 52) and would possess diminished enzymatic activity when compared to either the recombinant (32, 33) or natural (53) gelatinase A, which are devoid of TIMP-2. In this study, we showed that preincubation of progelatinase B with equal molar amounts of either TIMP-1 or TIMP-2 inhibits activation by the gelatinase A active species, as previously reported for activation of progelatinase B with stromelysin-1 (54). The effect of TIMP-1 may be due to formation of a complex with progelatinase B (6), whereas that of TIMP-2 may be caused by an inhibition of gelatinase A (32, 33), as shown in Table 1 with the M_r 45,000 species. Although the M_r 45,000 form of gelatinase A is less susceptible to inhibition by TIMP-2, compared to the M_r 62,000 species,⁴ the amount of TIMP-2 used in the experiment described in Table 1 was 10-fold higher than that of the M_r 45,000 species. At this concentration, TIMP-2 inhibits the activity of the M_r 45,000 species completely, as reported previously for the truncated forms of gelatinase A lacking the COOH-terminal domain (32). The inhibitory effect of TIMP-2 on progelatinase B activation was also demonstrated by inhibiting the plasma membrane and APMA activation of progelatinase A with TIMP-2. This is consistent with the ability of this inhibitor to form a complex with progelatinase A that prevents activation (33, 51, 55).

We have shown in this report that activation of human recombinant progelatinase A, with either plasma membrane or APMA, generates a M_r 62,000 and a M_r 45,000 species, as reported previously (25, 27, 32, 33, 55). Howard *et al.* (56) also reported the formation of a M_r 42,500 active gelatinase A fragment after separation of TIMP-2 from progelatinase A by acid treatment and reverse phase-HPLC. The presence of these species was also reported in the medium of fibroblast cells

treated with concanavalin A (25, 29, 30). The formation of the M_r 45,000 species, as shown in the present report, involves cleavage of both the NH₂-terminal profragment and the COOH-terminal domain. This was demonstrated by NH₂-terminal sequencing of the purified M_r 45,000 species that revealed an NH₂ terminus starting from Tyr⁸¹. The same residue was reported as the NH₂ terminus for the M_r 62,000 species after APMA treatment (56, 57) or plasma membrane activation of progelatinase A (27). In addition, a polyclonal antibody generated against the COOH-terminal end of progelatinase A failed to react with the M_r 45,000 species but reacted with the M_r 62,000 form, further demonstrating the absence of the COOH-terminal region in the M_r 45,000 species.

We isolated the active species of gelatinase A and showed that both the M_r 62,000 and the M_r 45,000 species activated purified progelatinase B. The ability of the 45-kDa species to activate progelatinase B is consistent with previous studies that showed that the COOH-terminal domain of gelatinase A is not required for catalytic activity (32, 37, 38). The purified M_r 62,000 and M_r 45,000 species were also able to activate progelatinase B present in the conditioned media of HT1080 cells. However, high levels of the exogenous gelatinase A species were required, possibly due to the presence of TIMPs in the media. Previous studies showed that activation of progelatinase A in the media of HT1080 cells that had been treated with phorbol ester did not cause the activation of the M_r 92,000 enzyme (26, 27). The lack of progelatinase B activation under these conditions may be due to the presence of TIMP-1, which is induced by the treatment with phorbol ester.⁴ Thus, the high levels of TIMP-1 results in inhibition of progelatinase B activation, and by consequence, a general inhibition of MMP activity ensues. This is consistent with our previous data showing the inhibitory effect of phorbol ester on the *in vitro* invasion of HT1080 cells (58).

The activation of progelatinase B by gelatinase A may be significant in tissues where stromelysin-1 may not be present (43). Recent *in situ* hybridization studies in 17 cases of breast cancer showed high levels of gelatinase A and B mRNAs in 60–80% of the cases, whereas low levels of stromelysin-1 mRNA were detected in only 30% of the cases.⁵ Also, our previous immunohistochemical studies showed a colocalization of gelatinase A and B in the malignant cells in 72% of 83 cases of breast carcinoma tissue (46). Other studies also showed coexpression of gelatinases in breast (44, 45), colon (10), and bladder cancers (14). Therefore, it is highly plausible that colocalization of

⁴ Unpublished results.

⁵ L. Matrisian, personal communication.

these enzymes in tumor tissue would facilitate their interaction for activation, leading to degradation of ECM.

Fig. 9 depicts a model for the interaction of several members of the MMP family, leading to the activation of progelatinase B. The membrane enzyme, MT-MMP, of tumor cells activates progelatinase A to generate the M_r 62,000 and M_r 45,000 species. This process has been recently shown to involve the binding of TIMP-2 to MT-MMP, followed by binding of progelatinase A to the TIMP-2-MT-MMP complex (55). The activation process and substrate specificity of MT-MMP remain to be defined. The active gelatinase A species may in turn activate progelatinase B, as shown in this study, and degrade ECM. Although the physiological significance of the M_r 45,000 species of gelatinase A (Fig. 9, *italics*) remains presently obscure, it is interesting that the rate of TIMP-2 inhibition of active gelatinase A species lacking the COOH-terminal domain is significantly lower than that of the M_r 62,000 form (32, 37, 38, 59). Thus, cleavage of the COOH-terminal domain, should it occur *in vivo*, would result in the formation of gelatinase A species that are less susceptible to TIMP-2 inhibition but remain catalytically competent and capable of activation of progelatinase B. Recently, activation of progelatinase B in fibroblast monolayers supplemented with plasminogen or prostromelysin-1 was described (40). Thus, stromelysin-1 would efficiently activate progelatinase B (39, 40, 54), if it were available; however, its expression in breast cancer remains unclear at the present (43). Plasmin, on the other hand, is a poor activator of progelatinase B (40, 41, 54). Nevertheless, the role of each proteinase in activation of progelatinase B would be determined by its abundance and accessibility, the state of activation, the rate of formation of the active species, and the presence of specific inhibitors (29). Although the role of TIMP-1 and TIMP-2 is not depicted in this model (Fig. 9), these inhibitors are critical regulators of the activation process and modulators of the enzymatic activity of the MMPs. As such, they play a crucial role in determining the extent of ECM degradation during tumor cell invasion. This and previous studies have shown that both TIMP-1 and TIMP-2 can inhibit the activation of progelatinase A and B and the respective proteolytic activity of the resultant enzymes by forming a complex with the latent enzymes and/or binding to the active enzyme forms. Further studies on the relationship between these MMPs and TIMPs will provide a clearer understanding of the regulation of MMP activity and the role of these proteases in tumor cell invasion.

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THE EFFECT OF PLATELETS ON INVASIVENESS AND PROTEASE PRODUCTION OF HUMAN MAMMARY TUMOR CELLS

Catherine BELLOC¹, He LU¹, Claudine SORIA¹, Rafael FRIDMAN², Yves LEGRAND¹ and Suzanne MENASHI^{1,3}

¹Unité 353 INSERM, Hôpital Saint-Louis, 1 Ave Claude Vellefaux, 75010 Paris, France; ²Department of Pathology, Wayne State University, Detroit, MI USA.

Interaction of tumor cells with platelets facilitates metastasis of tumor cells. It has been proposed that platelets protect tumor cells against the host's immune defense and enhance tumor-cell extravasation. In the present work we show that platelets increase the invasiveness of 3 mammalian cell lines (MCF-7, ZR-51 and MDA-MB231) through extracellular matrix, and propose this as an additional mechanism by which platelets facilitate metastasis. Since gelatinase and urokinase have both been implicated in degradation of the extracellular matrix and cell migration, and therefore in tumor invasion, we have also analyzed whether the interaction of platelets with tumor cells can modify the secretion of these proteases by tumor cells. MDA-MB231, which was the most invasive cell line among the 3 tested and was the most potent in inducing platelet aggregation, secreted the highest level of urokinase and was the only one in which gelatinase was detected. While platelets had no significant effect on the urokinase activity expressed by these cells, they induced in MDA-MB231 an important increase in the secretion of gelatinase, which can be reproduced by both platelet membrane and platelet releasate of activated platelets. This increase in gelatinase could be responsible, at least in part, for the increased invasiveness of these cells, since added TIMP-1 significantly reduced the number of cells which traversed matrigel.

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The relationship between malignancy and thrombotic disorder has long been recognized and thrombo-embolic disorders are an important cause of morbidity and mortality in malignant disease. Drugs that interfere with platelet function have been shown to limit metastasis production (Honn *et al.*, 1983, 1992). Platelets could essentially facilitate all steps, from initial tumor cell lodgement in the microvasculature to tumor-cell proliferation after arrest, interaction with the subendothelial matrix and extravasation. These diverse effects may result from direct platelet bonding with tumor cells or from the release reaction products following platelet aggregation and activation (for review, see Honn *et al.*, 1992).

Numerous studies have shown that tumor cells *in vitro* can induce platelet aggregation and this ability to aggregate platelets was correlated with their ability to cause metastasis *in vivo* (Pearlstein *et al.*, 1980). The mechanisms of induction of platelet activation appear to vary in the different tumor cells. The generation of thrombin could be responsible in most cases as tumor cells can express an activator of factor Xa and/or tissue-factor-like material (for review, see Gordon, 1992). Other mechanisms may also contribute, such as the activation of platelets by ADP produced by the tumor cells themselves or released by platelets in contact with tumor cells (Bastida *et al.*, 1982), and the production of 12 hydroxyeicosatetraenoic acid (12 HETE), which facilitates the expression of the glycoprotein IIb-IIIa receptor (Steinert *et al.*, 1993).

Platelet activation by tumor cells is thought to protect tumor cells from immune surveillance, to enhance tumor-cell adhesion to vascular endothelium—the first step of extravasation—and to provide permeability factors and tumor-growth factors which would facilitate extravasation and development of tumor metastasis (for review, see Gasic, 1984; Honn *et al.*, 1992). In this work, we studied the effect of platelets on the production, by tumor cells, of proteases which are implicated

in the degradation and invasion of the extracellular matrix (ECM). Both urokinase-type plasminogen activator (u-PA) and gelatinases have been implicated. u-PA activates plasminogen into plasmin, a wide-range serine protease, which degrades some of the components of the ECM either directly or indirectly by activating prometalloproteases (He *et al.*, 1989). Numerous studies have shown a correlation between tumor aggressiveness and expression of u-PA (for review, see Schmitt *et al.*, 1992). Gelatinase A (MMP-2) and gelatinase B (MMP-9) are metalloproteases of 72 and 92 kDa respectively, and numerous studies have implicated both of these in the digestion of basement membrane type-IV collagen in cancer (Ura *et al.*, 1989) and angiogenesis (Gross *et al.*, 1983). A correlation between tumor secretion of gelatinases (Ura *et al.*, 1989; Liotta and Stetler-Stevenson, 1989) and experimental metastasis has been reported. Transfection of gelatinase-B expression vector conferred metastatic capacity upon non-metastatic rat cells (Bernhard *et al.*, 1994). We have therefore compared the potential of 3 different mammary tumor cells (MCF-7, ZR 51 and MDA-MB231) to aggregate platelets, and the effect of platelets on the secretion of both u-PA and gelatinases by the 3 different types of tumor cells as well as their invasiveness through an ECM.

MATERIAL AND METHODS

Preparation of platelets

Platelets from human blood freshly collected in the anticoagulant acid-citrate dextrose were washed under sterile conditions as described by Patscheke and Worner (1978), in the presence of 100 ng/ml prostaglandin E₁. The platelets were finally resuspended in DMEM/F12 culture medium (GIBCO, Paisley, UK).

Activation of platelets

Platelet suspensions were incubated at 37°C for 15 min before thrombin was added at 0.2 U/ml and the suspension was agitated gently for 3 min to allow the aggregation to proceed. Thrombin was neutralized by the addition of 10 IU/ml hirudin (Sigma, Saint Louis, MO) and the aggregated platelets were then pelleted by centrifugation at 2,500 g for 10 min. The supernatant was used as a source of platelet-secreted factors and the pellet containing the degranulated platelets was resuspended in DMEM/F12 culture medium (GIBCO).

Cell lines and culture conditions

The human adenocarcinoma cell lines MDA-MB231 (Keydar *et al.*, 1978), MCF-7 (Soule *et al.*, 1973) and ZR-75-1

³To whom correspondence and reprint requests should be sent, at Unité 353 INSERM, Hôpital Saint-Louis, 1 Ave Claude Vellefaux, 75010 Paris, France. Fax: 33 1 42490733.

Abbreviations: TIMP, tissue inhibitor of metalloproteinases; PA, plasminogen activator; u-PA, urokinase-type plasminogen activator; PAI, plasminogen activator inhibitor; ECM, extracellular matrix.

(Engel and Young, 1978) (gifts from Dr. F. Calvo, IGM, Hôpital Saint-Louis, France) were cultured in DMEM/F12 containing 10% FCS (Biological Industries, Beth Haemek, Israel), 100 IU/ml of penicillin (GIBCO), 100 mg/ml of streptomycin (GIBCO) and glutamine (GIBCO) 200 mM. They were maintained under an atmosphere of 5% CO₂ at 37°C.

At confluence, tumor cells were washed to remove serum and then incubated in serum-free medium with or without a suspension of 2×10^8 platelets for 18 hr. In some cases platelet-released factors or degranulated platelets were used.

Platelet aggregation studies

Platelets were isolated from blood of healthy volunteers collected in the anticoagulant acid-citrate dextrose and washed as described by Patschke and Worner (1978). The platelets were suspended in a Tyrode buffer at 3×10^8 /ml. Tumor cells were detached from culture plates using EDTA 0.02%, centrifuged and resuspended in PBS. Aggregation was monitored in an aggregometer (Chrono Log, Haverton, PA) with constant stirring at 1,000 rpm. To 200 µl of tumor-cell suspension (0.4×10^6 cells/ml) were added 150 µl of washed platelet suspension and either 50 µl saline or 50 µl normal citrated plasma diluted 1/5 in saline. Platelet aggregation was also carried out in the presence of 10 IU/ml of hirudin (Sigma). Aggregation was recorded as a decrease in absorbance.

Gelatin zymography

SDS-gelatin gels were prepared as described by Herron *et al.* (1986). Briefly, 1 mg/ml of gelatin was incorporated into the standard Laemmli SDS-10% polyacrylamide gels. Conditioned medium was mixed with 2 vol of sample gel buffer (10% SDS, 30% glycerol, 0.25 M Tris-HCl, pH 6.8, 0.1% bromophenol blue). After electrophoresis, the gels were washed twice for 30 min each time in 2.5% Triton X-100 to remove SDS, incubated for 48 hr in 50 mM Tris buffer, pH 7.5, containing 5 mM CaCl₂, and then stained in 2% Coomassie brilliant blue. Clear zones of gelatin lysis against a blue background stain observed after destaining indicated the presence of enzyme. The molecular weight of the enzyme was determined in relation to standard proteins from 45 to 200 kDa MW (SDS-PAGE standard high range, Bio-Rad, Richmond, CA) run at the same time.

Western blot

Conditioned medium was electrophoresed on SDS polyacrylamide 10% gels and electrophoretically transferred to nitrocellulose. Blots were incubated for 1 hr at 22°C with Tris-tween buffer containing 1.5% BSA, then for 2 hr at 22°C with 5 µg/ml monoclonal antibody (MAb 209) raised against human pure recombinant gelatinase B. Bands were visualized by incubation with anti-mouse Ig-peroxidase hybridoma screening reagent (Boehringer Mannheim, Germany) at 1/2,000 dilution for 1 hr at 22°C and a chemiluminescence reaction (ECL Western blotting, Amersham, Aylesbury, UK).

Zymographic analysis of u-PA

This was performed according to the method of Granelli-Piperno and Reich (1978). After SDS-PAGE, gels were washed in 2.5% Triton X-100 followed by further washes in water. The gels were then applied to a plasminogen-rich fibrin agarose underlay containing 1.25% agarose in PBS, 2 mg/ml of fibrinogen (Kabi Vitrum, Stockholm, Sweden), 5 µg/ml of plasminogen (Chromogenix, Toulouse, France) and 0.16 IU/ml of thrombin. After incubation for 16 hr at 37°C in a moist atmosphere, lysis zones were visible. Controls were performed with plasminogen-free fibrin-plates.

Spectrophotometric PA assays

The u-PA activity was determined both in cells and in conditioned medium. To 96-well plates were added 100 µl conditioned medium or 100 µl suspension of cells, 50 µl PBS

containing 1 mg/ml BSA, 50 µl plasminogen (500 µg/ml) (or 50 µl buffer for control) and 50 µl of plasmin-sensitive substrate S2251 (Chromogenix) (3 mM). To differentiate between t-PA and u-PA, amidolytic activity on the cells was also performed in the presence of 1 mM amiloride (Sigma). For culture medium, amidolytic assays were performed in both the absence and the presence of 10 µl of 5 mg/ml fibrin monomers. After various incubation times (15 min–1 hr) at 37°C, the absorbance at 405 nm was read in a Dynatech (Chantilly, VA) ELISA reader. Purified u-PA (Hoechst, Puteaux, France) was used as a standard. Results were expressed in u-PA U/10⁶ cells.

Invasion assay

The invasion assay was performed as previously described (Frandsen *et al.*, 1992). Briefly, polycarbonate filters of 12 µm pore size (Costar, Cambridge, MA) were coated with 50 µg/filter of an extract of basement membrane components Matrigel (a gift from Dr. J. Foidart, University of Liège, Belgium) and placed in a Boyden chamber. Tumor cells detached with 0.02% EDTA were resuspended in DMEM/F12 medium containing 0.2 mg/ml BSA, then 2×10^5 cells were added to each filter in the upper compartment of the Boyden chamber. Fibroblast-conditioned medium containing 2 mg/ml BSA was placed in the lower compartment as a source of chemoattractants. After incubation for 24 hr at 37°C, cells that invaded the lower surface of the filters were fixed with methanol, stained with H. and E. and counted. The data are expressed as the total number of cells per microscopic field. Ten fields were counted in each of 3 different experiments.

RESULTS

1. Platelet aggregation induced by the 3 mammary-tumor cell lines

All 3 cell lines caused platelet aggregation when added to platelet suspension in Tyrode buffer, provided that platelet-poor plasma was present (Fig. 1), and the aggregation was stopped by clot formation. In all cases, a lag phase was present but the length of the lag phase depended on the number and type of tumor cells added to the platelet suspension. Using the same number of cells, MDA-MB231 induced platelet aggregation with the shortest lag phase. The platelet-aggregating activity of these 3 cell lines was due to the generation of thrombin, since the aggregating effect was completely inhibited by the addition of 5 IU/ml hirudin. These results suggest that plasma provided prothrombin which was converted into active thrombin by the tumor cells.

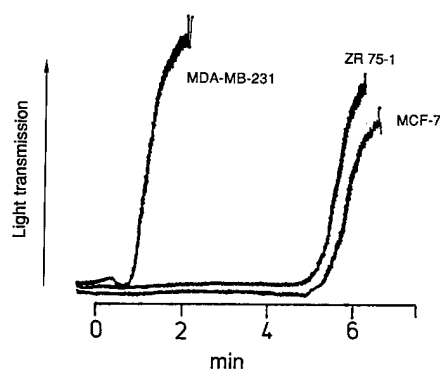


FIGURE 1 – Platelet aggregation induced by tumor cells. To 200 µl of tumor cells (0.4×10^6 /ml) were added 150 µl of washed platelet suspension (5×10^8 /ml) and 50 µl of plasma diluted 1/5 in saline. Platelet aggregation was recorded.

2. Production of gelatinases by the 3 mammary-cancer cell lines: effect of platelets

Zymographic analysis of the 3 cell lines (MDA-MB231, ZR 75-1 and MCF-7) showed that only MDA-MB231 cells secreted gelatinase activity. This activity at 92 kDa corresponding to gelatinase B was only detected in the conditioned medium and none was found in the cell lysate. No activity corresponding to the 72-kDa gelatinase A was detected. When MDA-MB231 cells were incubated with platelets for 24 hr, the intensity of the gelatinase-B lysis band greatly increased in the conditioned medium, whereas platelets did not induce any visible effect in the 2 other cell lines which did not secrete detectable gelatinase (Fig. 2a). No lysis band was observed when platelets alone were tested. The increase in the area of lysis of MDA-MB231 cells induced by platelets was not accompanied by a change in the molecular weight of the gelatinase, suggesting that this increase in lysis is related to an increased secretion rather than to an activation of the proenzyme. This increase in gelatinase secretion was confirmed by Western blot (Fig. 2b).

The secretion of gelatinase B by MDA-MB231 was studied using different matrix components as cell support, including collagen types I and IV, gelatin, fibronectin and laminin. While some variations were found in the basal secretion of gelatinase by the MDA-MB231 cells, depending on the matrix on which they were cultured, a similar increase in gelatinase activity was induced by platelets, regardless of the matrix which was used (results not shown).

In order to evaluate whether the effect of platelets is due to a soluble factor released into the supernatant during platelet activation or to the activated platelets themselves, we have compared the gelatinase secretion by MDA-MB231 cells incubated with either the platelet releasate or with the remaining degranulated platelets. As shown in Figure 3, the lysis band corresponding to the gelatinase was increased both by the supernatants of activated platelets and by the degranulated platelets.

3. Production of plasminogen activators by the 3 mammary-cancer cell lines: effect of platelets

All 3 cell lines expressed amidolytic activity which was only detected in the presence of plasminogen and therefore repre-

sented a true PA activity. This PA activity was of the urokinase type (u-PA) since it was totally inhibited by the presence of 1 mM amiloride, a selective inhibitor of u-PA (Sappino *et al.*, 1993). Therefore, the results were expressed as u-PA units. As shown in Table I, the highest u-PA activity was found in MDA-MB231 cells.

When the cells were treated for 24 hr with platelets, a small increase in u-PA activity was noted for MDA-MB231 cells and MCF-7. However, this increase was not statistically significant.

A very low level of u-PA activity was detected in the conditioned medium (less than 5% of the cell-associated u-PA), indicating that the u-PA secreted by these cells remained associated with the cell membrane. Platelets did not have a significant effect. Plasminogen-dependent amidolytic activity was not increased by the addition of fibrin monomers to the medium (results not shown), indicating that these cells did not secrete t-PA.

As platelets contain the u-PA inhibitor PAI-1, the possibility that this PAI-1 masks an increase in u-PA secretion brought about by platelets was investigated by zymography, which reveals both free u-PA and the complex u-PA/PAI. Figure 4 shows the zymography of PA activity obtained with the MDA-MB231 cell line. A major lysis band was observed at the region of 55 kDa which migrated to the same position as the high-molecular-weight urokinase, while the lysis band of 95 kDa corresponding to the u-PA/PAI-1 complex was very faint. The addition of platelets had no noticeable effect on the free u-PA band, but a small increase in the complex was observed. This increase in the u-PA/PAI complex was only observed with

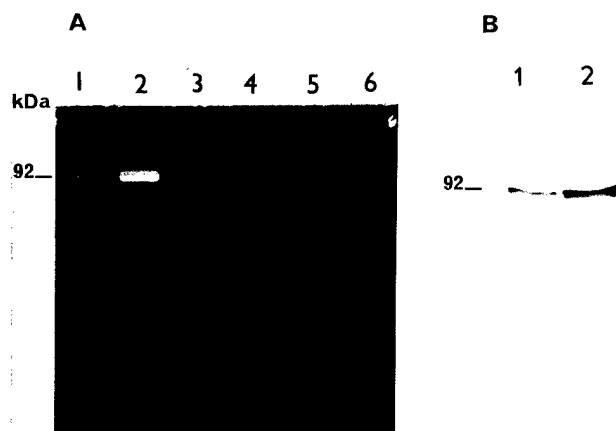


FIGURE 2 – Effect of platelets on the secretion of gelatinase B by tumor cells. After 24 hr incubation of the 3 different cell lines in serum-free medium without or with platelet suspension (2×10^8 /ml), conditioned medium was tested at cell confluence by: (a) gelatin zymography. Lanes 1–2: MDA-MB231; lanes 3–4: ZR 75-1; lanes 5–6: MCF-7. Lanes 1, 3, 5: without platelet and lanes 2, 4, 6 in the presence of platelets. (b) Immunoblot of gelatinase B produced by MDA-MB231. Lane 1 without platelets, and lane 2 in the presence of platelets.

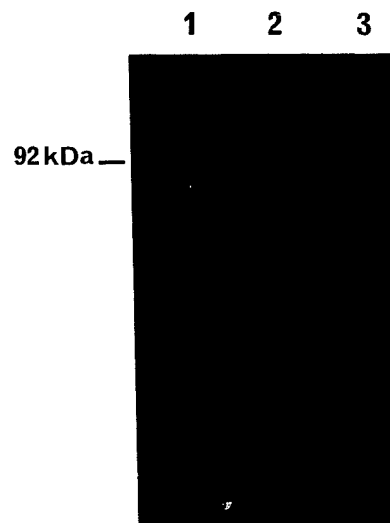


FIGURE 3 – Gelatin zymography: comparison between the effect of releasate of activated platelets and degranulated platelets on the secretion of gelatinase B by MDA-MB231. MDA-MB231 cells at confluence were incubated for 24 hr: Lane 1 in serum-free medium; lane 2 with supernatants of thrombin-activated platelets and lane 3 with platelet pellets obtained after thrombin activation and centrifugation at 15,000 g for 10 min. Thrombin was used at 0.2 U/ml and neutralized after aggregation by 10 IU/ml hirudin.

TABLE I – U-PA ACTIVITY (U/10⁵ CELLS) EXPRESSED BY THE 3 DIFFERENT MAMMARY CELL LINES: EFFECT OF PLATELETS

	MDA-MB231	MCF-7	ZR 75-1
Without platelets	21.9 ± 4.1	2.89 ± 0.8	1.19 ± 0.35
With platelets	27.7 ± 5.2	3.35 ± 0.5	1.20 ± 0.20

Results are expressed as mean ± SD of 4 different experiments.

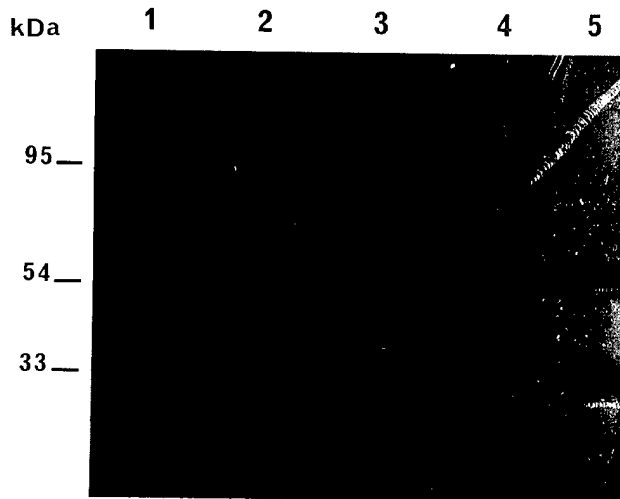


FIGURE 4 – Fibrin zymographic detection of the activity of cellular plasminogen activators (PA) on MDA-MB231: effect of platelets. MDA-MB231 at confluence were incubated for 24 hr. Lane 1, in serum-free medium; lane 2, with intact platelets; lane 3, with supernatants of thrombin-activated platelets; and lane 4, with platelet pellets obtained after thrombin activation and centrifugation at 15,000 g for 10 min (thrombin was used at 0.2 U/ml and was neutralized after aggregation by 10 IU/ml hirudin). Lane 5: purified u-PA.

the platelet releasate which contained the PAI and not with the degranulated platelets. Since this complex was not evidenced when purified uPA was added to platelet lysate which contains inactive PAI-1 (results not shown), we can only suggest that platelet PAI was activated by some factors secreted by the cells during the incubation thus able to form a complex with uPA.

The u-PA activity in the 2 other cell lines was very low and not modified by platelet addition.

Invasiveness of the 3 cell lines through the matrigel

MDA-MB231 cells, which secreted the highest levels of both u-PA and gelatinase, showed the greatest capacity to traverse the matrigel in the Boyden chamber (Fig. 5). When platelet releasate was added to the cells, the capacity to traverse the matrigel was greatly increased ($p < 0.001$). Since platelets also increased gelatinase secretion in MDA-MB231 cells, the participation of this protease in matrigel invasion was evaluated by the addition of the metalloproteinases inhibitor TIMP-1 (2 μ g/ml). An inhibition of 60% was observed, suggesting a possible role for gelatinase in the platelet-induced invasiveness of the MDA-MB231 cell line, although the participation of other metalloproteinases, such as stromelysin, cannot be ruled out. The fact that the matrigel invasion by the 2 cell lines, ZR 75-1 and MCF-7, which do not secrete detectable gelatinase, was also increased in the presence of platelets, indicates that other factors are involved.

DISCUSSION

Tumor cells in the vasculature are frequently observed in association with platelets, and this association has been postulated to be essential for the accomplishment of metastasis. The interaction of tumor cells with the platelets is thought to protect the cells against immune host defense mechanisms in the blood and to favor tumor-cell extravasation both by enhancing cell adhesion to the endothelium and by inducing

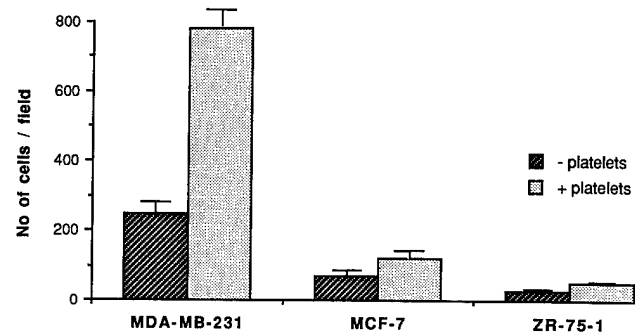


FIGURE 5 – Invasiveness of the tumor cells: effect of platelets. Tumor cells (2×10^5) were introduced into the upper compartment of the Boyden chamber without or with the supernatant of 3×10^7 thrombin-activated platelets in the presence of 0.1 mM APMA (4-aminophenylmercuric acetate), activator of metalloproteinases. After incubation at 37°C for 18 hr, the cells were counted on the lower side of the filter, after the cells remaining on the upper side had been scraped off. Results were expressed as the number of cells per microscopic field. Ten fields were counted in each of 3 different experiments.

endothelial cell retraction (Honn *et al.*, 1992). This present work suggests an additional role for platelets in the development of metastasis by facilitating the invasiveness of tumor cells through the ECM. Platelets significantly increased the invasiveness of the 3 types of mammalian tumor cell tested (MDA-MB231, MCF-7 and ZR-51). This increased invasiveness is due, at least partially, to an increase in gelatinase secretion. This was shown in the case of MDA-MB231 where the basal level of gelatinase was greatly stimulated by platelets. The stimulation of gelatinase shown by zymography corresponded to an increase in the protein level, demonstrated by Western blot, thus indicating increased secretion of the protease and not activation of the proenzyme. However, platelet-induced gelatinase could not be shown with the other 2 cell lines which did not produce detectable basal levels of gelatinase. This suggests that, in addition to gelatinase, platelets increase invasiveness of tumor cells by some other mechanisms. This is also supported by the fact that TIMP-1, a specific inhibitor of metalloproteinase, did not totally block the platelet-induced invasiveness (60% inhibition) of MDA-MB231. We can exclude a role for cell-associated u-PA as a mechanism of platelet-induced invasiveness, since u-PA was not significantly modified by platelets in any of the 3 cell lines, whatever the basal value.

The mechanisms proposed so far for the role of platelets in the metastatic process all refer to events occurring during the passage of tumor cells into the circulation. The increased invasiveness shown in this study suggests an additional role for platelets once tumor cells have traversed the endothelium into the ECM. We propose, therefore, a new mechanism by which the effect of platelets renders tumor cells more invasive, partially due to gelatinase secretion. We have already shown that platelets induce the secretion by endothelial cells of both gelatinase and u-PA (Menashi *et al.*, 1991), both implicated in angiogenesis. Taken together, these results suggest that platelets may act by favoring both tumor invasiveness and angiogenesis which is required for tumor progression and extravasation.

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Carbohydrate-Mediated Regulation of Matrix Metalloproteinase-2 Activation in Normal Human Fibroblasts and Fibrosarcoma Cells

David C. Gervasi,* Avraham Raz,*† Marie Dehem,* Maozhou Yang,‡
Markku Kurkinen,*‡ and Rafael Fridman*¹

*Department of Pathology, †Tumor Progression and Metastasis, Karmanos Cancer Institute, and

‡Center for Molecular Medicine and Genetics, Wayne State University, Detroit, Michigan 48201

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Matrix metalloproteinase-2 (MMP-2) is activated on the cell surface by membrane type 1-MMP (MT1-MMP). Activation of proMMP-2 is induced *in vitro* by concanavalin A (ConA). The regulation of proMMP-2 activation is, however, not yet fully understood. We investigated the effect of plant lectins, carbohydrates and inhibitors of the cytoskeleton on proMMP-2 activation in normal (HLF1) and malignant fibroblast (HT1080) cells. Native ConA induced proMMP-2 activation in both cell types while dimeric succinyl-ConA had no effect, suggesting that receptor clustering is involved in activation. Wheat germ agglutinin (WGA) also induced proMMP-2 activation. N-acetyl-D-glucosamine (GlcNAc) inhibited the effects of ConA and WGA while mannose only inhibited ConA-induced proMMP-2 activation. Mannose also inhibited the expression of MT1-MMP mRNA induced by ConA. Cytochalasin B and colchicine had no effect on the ConA induction of proMMP-2 activation. These studies help to define some of the cellular and molecular mechanisms for the induction of proMMP-2 activation. © 1996 Academic Press, Inc.

MMP-2 (gelatinase A, 72 kDa type IV collagenase) is a secreted endopeptidase able to hydrolyze several components of the extracellular matrix including basement membrane collagen IV and has been associated with tumor invasion and metastasis (1,2). MMP-2 also degrades non-collagenous proteins like galectin-3 (3), β -amyloid (4) and fibroblast growth factor receptor-1 (5). Thus, the broad specificity of MMP-2 may play a role in the regulation of various cellular activities. Like other members of the MMP family, MMP-2 is secreted in a latent form that is activated by a sequential cleavage of the N-terminal propeptide domain resulting in the generation of two active species of 62 and 59 kDa (6) and activation is cell surface-dependent (6-8). This process could be induced in cultured cells by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (8), ConA (7), transforming growth factor- β (8) and a collagen substrate (9). Analysis of the activating factor in the plasma membranes resulted in the identification of a novel type of MMP, the membrane type-MMP (MT-MMP) (10, 11). MT-MMPs are a subclass of MMPs that contain a unique transmembrane domain and a RXXR motif downstream of the N-terminal propeptide that is recognized by furin-like enzymes and may serve as a cleavage site for intracellular activation. Currently, four different MT-MMPs were isolated from normal and tumor tissues (10,12-14). However, the specific role of each type of MT-MMP in MMP-2 activation remains to be determined.

An important event in the understanding of the cellular mechanisms involved in proMMP-2 activation was the discovery by Overall and Sodek (15) of the ability of ConA to modulate the expression and activation of MMP-2 in human fibroblast cells. ConA is a widely used plant lectin composed of four identical subunits that specifically binds to cell

¹ All correspondence should be addressed to: Dr. Rafael Fridman, Department of Pathology, Wayne State University, 540 E. Canfield Avenue, Detroit, MI 48201. Fax: (313) 577-8180. E-mail: rfridman@med.wayne.edu.

surface glycoproteins containing branched mannose residues. In mammalian cells, binding of ConA to cell surface glycoconjugates produces a variety of cellular responses including agglutination, cell proliferation, and clustering and internalization of cell surface receptors. In many aspects, the responses induced by ConA mimic the action of physiologically relevant ligands. ConA has been used as an important tool for defining some of the cellular mechanisms involved in the activation of MMP-2. For example, ConA-induced activation of proMMP-2 has been shown to require protein synthesis (16). In addition, ConA enhances the expression of MT1-MMP mRNA in breast (16) and cervical (17) cancer cells and MMP-2 mRNA in primary human fibroblasts (15). Like ConA, TPA induces MMP-2 activation in a variety of cultured cells (18). In the present study, we utilized lectins and TPA to further define some of the factors required for MMP-2 activation in HLF1 and HT1080 cells. Specifically, we investigated the carbohydrate specificities and involvement of the cytoskeleton in the activation of proMMP-2 induced by ConA and TPA and their effect on MT1-MMP mRNA and protein expression in both cell types.

MATERIALS AND METHODS

Cell culture. Human HT1080 fibrosarcoma (CCL-121) cells and normal human lung HLF1 fibroblasts (CCL-153) were obtained from American Type Culture Collection (Rockville, MD). The cell lines were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum and antibiotics and maintained in a 5% CO₂ incubator at 37°C.

Reagents and chemicals. ConA, WGA, succinyl-ConA, TPA, cytochalasin B, colchicine, D(+) mannose, N-acetyl-D-glucosamine (GlcNAc), N-acetyl-D-galactosamine (GalNAc), and D-galactose were all purchased from Sigma Co (St. Louis, MO). The lectins were all dissolved in serum-free DMEM containing 25 mM Hepes. The TPA stock solution was made in ethanol to a final concentration of 2 mM. Cytochalasin B and colchicine stock solutions were prepared in dimethyl sulfoxide (DMSO) to final concentration of 2 and 2.5 mM, respectively.

Cell treatments. Cells were grown to 80% confluence in 24-well plates in complete media. At the time of treatment, the growth media was removed and the cells were washed twice with warm serum-free DMEM. Then, 0.3 ml/well of serum-free DMEM containing various concentrations of lectins or TPA (100 nM final concentration) were added to the cells for a 16 hr incubation period. In the case of TPA, the final concentration of ethanol added to the cells was 0.005% or 0.05 μ l/ml. With cytochalasin B and colchicine the final concentration of DMSO was 0.95 and 0.08% (v/v), respectively. In each case, the same amount of vehicle was added to control cultures. The media was then collected, briefly centrifuged (14,000 rpm, 5 min) to remove cell debris and immediately analyzed by gelatin zymography. After each treatment, cell viability was determined by trypan blue exclusion.

Zymography. Gelatin-zymography was performed using 10% SDS-polyacrylamide gels containing 1% gelatin (18). Briefly, supernatants (15-20 μ l) were mixed with 4X Laemmli sample buffer without reducing agents and without heating and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). At the end of the electrophoretic run, the gels were incubated (20 min., 22°C) in a solution of 2.5% Triton-X100 in H₂O and then washed in distilled H₂O, for another 20 min. The gels were then incubated (16 hrs, 37°C) in 50 mM Tris-HCl, 5 mM CaCl₂, pH 8 and then stained with 0.25% Coomassie Blue in a solution of 10% methanol and 5% acetic acid. Bands of gelatinolytic activity detected as cleared bands against the blue-stained gelatin background were visualized after destaining the gels with 10% methanol-5% acetic acid.

Northern blot analysis. Total RNA was extracted from untreated or treated HT1080 and HLF1 cells using the RNeasy Total RNA kit (Qiagen, Chatsworth, CA). Five μ g RNA from each sample were fractionated on a 1% agarose gel in the presence of formaldehyde and then transferred onto a nylon membrane (Hybond-N, Amersham, Aylesbury, UK) followed by fixation to the membrane using an optimized UV crosslinking procedure. The blot was prehybridized at 42°C for 3 hr followed by hybridization at 42°C for 18 hr with a ³²P-labeled human cDNA probe for MT1-MMP (1.75 kb). Then, the blot was washed twice (15 min. each) with 0.1X SSC-0.1% SDS at 65°C and autoradiographed at -80°C. RNA loading was normalized using the signal obtained with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe. Quantitation of signals was performed using an AmbisTM Radioanalytic Imaging System.

Immunoblot analysis. Analysis of MT1-MMP protein expression was performed in untreated and treated HT1080 and HLF1 cells extracted with the byphasic detergent Triton X-114. Briefly, cells were lysed with 1.5% Triton X-114 in Tris buffered saline (TBS, 50 mM Tris-HCl pH 7.5, 150 mM NaCl) containing 1 mM CaCl₂, 1 mM MgCl₂, 1 mM phenylmethylsulfonylfluoride (PMSF) and 5 mM EDTA. The cell extracts were then briefly (5 min) centrifuged (14,000 rpm, 4°C), and the supernatants were warmed (2 min., 37°C) and centrifuged (14,000 rpm, 22°C) to separate the detergent and aqueous phases. The detergent phase of each sample was diluted (4-fold) with distilled water

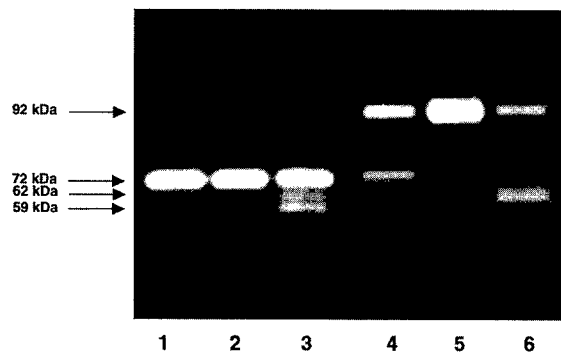


FIG. 1. Effect of Con and TPA on proMMP-2 activation in HT1080 and HLF1 cells. HLF1 (lanes 1-3) and HT1080 (lanes 4-6) cells were untreated (lanes 1 and 4) or treated (12 hrs) with either TPA (100 nM) (lanes 2 and 5) or ConA (10 μ g/ml) (lanes 3 and 6) in serum free media. The media was then collected and analyzed by gelatin-zymography. The molecular weights in the left represent the apparent molecular mass of latent and active MMP-9 (92 kDa) and MMP-2 (62 and 59 kDa) forms.

followed by the addition of sample buffer. The samples were then subjected to SDS-PAGE and transferred to BA-S 85 nitrocellulose membrane (Schleicher & Schuell, Keene, NH). Blots were blocked with 3% bovine serum albumin and 3% nonfat dry milk in 100 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.02% NaN_3 (blotto). The blots were then incubated with a polyclonal rabbit antibody raised against a synthetic peptide (RFNEELRAVDSEYPNIK) derived from the amino acid sequence of human MT1-MMP (10) and produced by Genetics Research, Inc. (Huntsville, AL) based on antigenic site predictions of Dr. Steve Ledbetter (Genzyme, Cambridge, MA). The blots were incubated with anti-MT1-MMP antibody diluted (1:5000) in 20 mM Tris-HCl, pH 7.5, containing 137 mM NaCl and 0.1% Tween-20 and then incubated with a goat anti-rabbit polyclonal antibody. The immunodetection of the antigen was performed using the ECL kit (Amersham) according to the manufacturer's instructions.

RESULTS

Effect of ConA and TPA on proMMP-2 activation. We initially confirmed the ability of ConA and TPA to induce activation of proMMP-2 in HT1080 and HLF1 cells. As shown in Figure 1, ConA induces proMMP-2 activation in HLF1 cells resulting in the generation of the 62- and 59-kDa forms (Figure 1, lane 3) confirming previous reports (6,18) while TPA had no effect (Figure 1, lane 2). TPA treatment of HT1080 cells, which constitutively produce both proMMP-9 and proMMP-2 (Figure 1, lane 4), resulted in the secretion of higher amounts of proMMP-9 (19) and the appearance of the active 62-kDa form of MMP-2 (Figure 1, lane 5). Dose dependent experiments demonstrated a maximal activation of proMMP-2 at ConA concentrations of 5-10 μ g/ml for both HLF1 (Figure 2, lanes 5 and 6) and HT1080 cells (Figure 3, lane 3). Higher doses of ConA (20-30 μ g/ml) did not alter the pattern of proMMP-2 activation. It should be noted that with any given dose of ConA, conversion of the latent form to the active species was never complete after a 24 hr incubation period.

Effect of Succinyl-ConA. Succinyl-ConA is a dimeric protein lectin that maintains the carbohydrate binding specificity of native ConA but does not induce ligand mobility or clustering on the cell surface. We compared the effects of ConA and succinyl-ConA on proMMP-2 activation in HLF1 and HT1080 cells. As shown in Figures 2 and 3, treatment of cells with succinyl-ConA for 24 hrs consistently failed to induce a detectable proMMP-2 activation in both cell types even at doses as high as 25 μ g/ml. This suggests that clustering of cell surface mannose-containing glycoproteins are required for the ConA-induced activation of proMMP-2 in both cell types.

Effect of WGA and sugars on proMMP-2 activation. We wished to define some of the cell surface carbohydrate moieties involved in the signalling process leading to proMMP-2 activa-

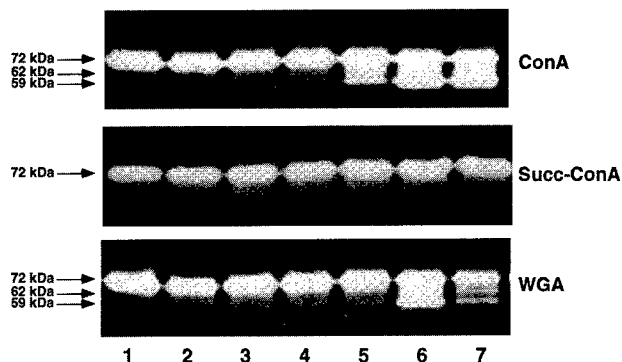


FIG. 2. Effect of ConA, Succinyl-ConA and WGA on proMMP-2 activation in HLF1 cells. HLF1 cells were treated (12 hrs) with increasing concentrations (lane 1: O; lane 2: 0.62; lane 3: 1.25; lane 4: 2.5; lane 5: 5; lane 6: 10 and lane 7: 20 $\mu\text{g/ml}$) of either ConA, succinyl-ConA or WGA in serum free media. The media was then collected and subjected to gelatin-zymography as described in Materials and Methods. The molecular weights represent the apparent molecular mass of latent and active MMP-2 forms.

tion by ConA. To this end, we compared the effects of ConA and WGA which differ in their carbohydrate recognition specificities. ConA binds α -mannose, glucose and α -GlcNac and WGA binds β -GlcNac and sialic acid. In addition, we tested the ability of purified sugars to block the effect of these lectins on proMMP-2 activation. As shown in Figures 2 and 3, exposure of HLF1 (Figure 2) and HT1080 cells (Figure 3) to WGA (5-20 $\mu\text{g/ml}$) resulted in the activation of proMMP-2 in pattern similar to that obtained with ConA. Addition of mannose in the presence of 10 $\mu\text{g/ml}$ ConA to HT1080 cells lead to a significant reduction in the amount of the 62-kDa form and inhibition of formation of the 59-kDa species (Figure 4, lanes 3-6, upper panel). At 400 nM, mannose caused a non-specific reduction in the secretion of both MMPs. Addition of GlcNac in the presence of ConA (Figure 4, lanes 7-9, upper panel) also inhibited proMMP-2 activation. Mannose had no apparent effect on the WGA-induced

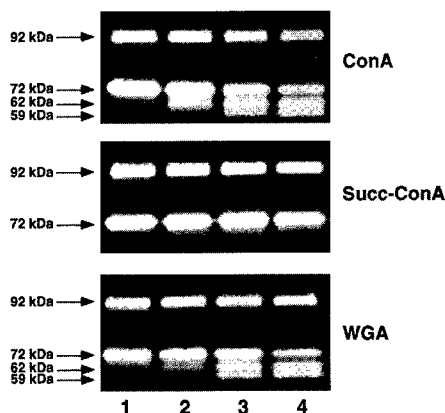


FIG. 3. Effect of ConA, Succinyl-ConA and WGA on proMMP-2 activation in HT1080 Cells. HT1080 cells were treated (12 hr) with, lane 1:0; lane 2:5; lane 3: 10 and lane 4: 20 $\mu\text{g/ml}$ of either ConA, succinyl-ConA or WGA in serum free media. The media was then collected and subjected to gelatin-zymography as described in Materials and Methods. The molecular weights represent the apparent molecular mass of latent and active MMP-9 and MMP-2 forms.

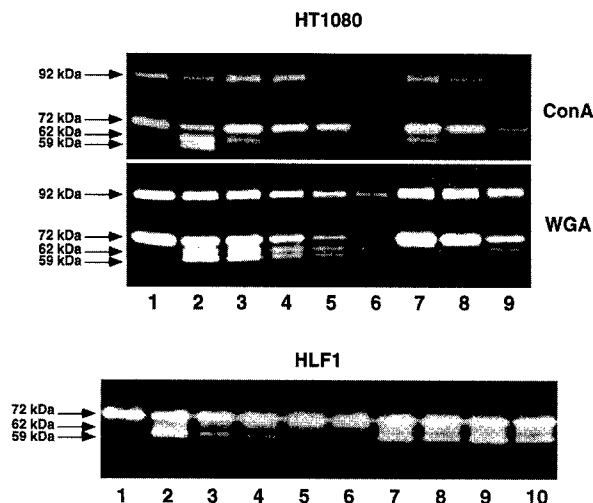


FIG. 4. Effect of mannose, GlcNac, GalcNac and galactose on proMMP-2 activation induced by ConA and WGA. HT1080 cells were treated with serum free media containing ConA (10 $\mu\text{g}/\text{ml}$) or WGA (10 $\mu\text{g}/\text{ml}$) (lanes 2-9) in the absence (lane 2) or presence of either mannose (lanes 3: 50 mM; lane 4: 100 mM; lane 5: 200 mM; lane 6: 400 mM) or GlcNac (lane 7: 50 mM; lane 8: 100 mM; lane 9: 200 mM). HLF1 cells were treated with ConA (10 $\mu\text{g}/\text{ml}$) (lanes 2-5 and 7-10) in the presence of either mannose (lane 3: 100 mM; lane 4: 200 mM; lane 5: 400 mM), GlcNac (lane 7: 200 mM; lane 8: 400 mM) or galactose (lane 9: 200 mM; lane 10: 400 mM). In lane 6, HLF1 cells received only 100 mM mannose. The media was then collected and subjected to gelatin-zymography as described in Materials and Methods.

proMMP-2 activation (Figure 4, lanes 3-6, lower panel). In contrast, GlcNac strongly inhibited the induction of proMMP-2 activation by WGA (Figure 4, lanes 7-9, lower panel). Similar inhibitory results were observed in HLF1 cells incubated with WGA (not shown) or ConA in the presence of mannose (Figure 4, lanes 3-5) or GlcNac (not shown). Treatment of HT1080 (not shown) or HLF1 (Figure 4) cells with ConA in the presence of GalcNac (Figure 4, lanes 7 and 8) or galactose (Figure 4, lanes 9 and 10) had no effect on activation of proMMP-2.

Effects of plant lectins and TPA on MT1-MMP expression. The activation of proMMP-2 induced by ConA and TPA has been previously shown to be mediated by MT1-MMP (10,11). Therefore, we examined the mRNA and protein expression of MT1-MMP in HLF1 and HT1080 cells treated with lectins (Con A or WGA) or TPA. As shown in Figure 5, both untreated HT1080 (lane 1) and HLF1 (lane 5) cells constitutively produce the 4.5 kb MT1-MMP mRNA. Exposure of HT1080 cells to TPA (Figure 5, lane 2) induced a 2-fold induction in MT1-MMP mRNA expression in agreement with a previous study (20). ConA induced a 1.5 fold expression over the basal levels and WGA had no significant effect (Figure 5, lanes 3 and 4, respectively). In HLF1 cells, Con A (Figure 5, lane 7) and WGA (Figure 5, lane 8) treatment resulted in a 3- and a 2.6 fold induction of MT1-MMP mRNA expression over basal level, respectively. The induction of MT1-MMP mRNA by ConA in HLF1 cells was abolished by 100 mM mannose (Figure 5, lane 9) consistent with the inhibitory effect of mannose on ConA-induced proMMP-2 activation (Figure 4). Interestingly, TPA treatment of HLF1 cells [which failed to induce proMMP-2 activation (Figure 1)], resulted in a 1.9 fold induction of MT1-MMP mRNA expression (Figure 5, lane 6). The expression of the MT1-MMP polypeptide was also examined by immunoblot analysis using a polyclonal antibody against a synthetic peptide derived from the human MT1-MMP sequence. This antibody reacts specifically with a protein of 63-kDa in purified plasma membranes of TPA-treated HT1080 cells (Figure 6, lane 1). Since MT1-

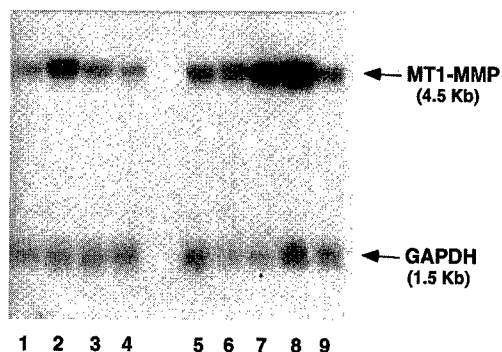


FIG. 5. Expression of MT1-MMP mRNA in HT1080 and HLF1 Cells. Cultures of HT1080 (lanes 1-4) and HLF1 (lanes 5-9) cells were treated with either 100 nM TPA (lanes 2 and 6), 10 µg/ml ConA (lanes 3 and 7), 10 µg/ml WGA (lanes 4 and 8) or 10 µg/ml ConA in the presence of 100 mM mannose (lane 9) in serum free media. Parallel cultures were established without treatment (lanes 1 and 6). After 16 hrs, total RNA was extracted, electrophoresed on a 1% agarose gel, blotted to nitrocellulose paper and probed with cDNA probes to MT1-MMP and GAPDH.

MMP is a plasma membrane-bound enzyme, we examined the expression of MT1-MMP in the detergent phase of Triton X-114 solubilized cells as described in Materials and Methods. This detergent extraction procedure resulted in a better resolution of the MT1-MMP protein. As shown in Figure 6, lane 2, extracts of untreated HLF1 cells did not show detectable levels of MT1-MMP protein, however, when the cells were treated with either ConA (Figure 6, lane 3) or WGA (Figure 6, lane 4), expression of MT1-MMP was readily detected. In HT1080 cells, expression of MT1-MMP was observed in untreated cells (Figure 6, lane 5) and its expression was apparently unaltered by treatment with ConA and WGA (Figure 6, lanes 6 and 7, respectively).

Effect of cytochalasin B and colchicine on ConA-induced activation of proMMP-2. We wished to test whether the integrity of the cytoskeleton plays a role on proMMP-2 activation after treatment with ConA. To this end, we examined the effects of cytochalasin B, which disrupt microfilament function and architecture, and colchicine, which disaggregates the microtubules. HLF1 and HT1080 cells (Figure 7, only HT1080 cells are shown) were treated (30

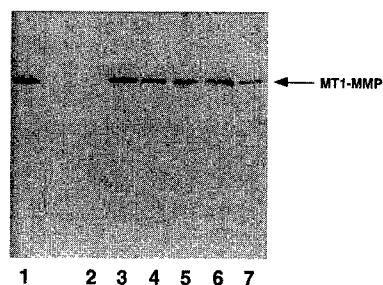


FIG. 6. Immunoblot analysis of MT1-MMP. HLF1 (lanes 2-4) and HT1080 (lanes 5-7) cells (2×10^5 cell/well) were either untreated (lanes 2 and 5) or treated with 10 µg/ml ConA (lanes 3 and 6) or 10 µg/ml WGA (lanes 4 and 7) for 16 hrs in serum free media. Cells were then lysed with 1.5% Triton X-114 in Tris buffered saline to obtain the detergent and aqueous phase as described in the Materials and Methods. The detergent phase of each sample was then subjected to SDS-PAGE in 12% polyacrylamide gels under reducing conditions followed by transfer to a nitrocellulose membrane. MT1-MMP (63 kDa) was detected using a polyclonal antibody and the ECL kit. Lane 1 shows the expression of MT1-MMP in purified plasma membranes of TPA-treated HT1080 cells.

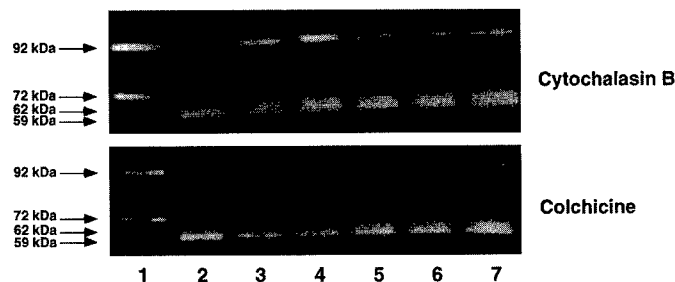


FIG. 7. Effect of cytochalasin B and colchicine in proMMP-2 activation. HT1080 cells were untreated (lane 1) or treated (30 min, 37°C) with various concentrations of either cytochalasin B (lane 3: 2.5 μ M; lane 4: 5 μ M; lane 5: 10 μ M; lane 6: 20 μ M) or colchicine (lane 3: 0.25 μ M; lane 4: 0.5 μ M; lane 5: 1 μ M; lane 6: 2 μ M) before addition of 10 μ g/ml of ConA (lanes 2-7). Lane 7 shows cells treated only with ConA and 0.08% (colchicine) or 0.95% (cytochalasin B) DMSO (final concentration), the vehicle of the two drugs.

min, 37°C) with various concentrations of either cytochalasin B or colchicine before addition of 10 μ g/ml ConA for another 16 hrs. The media was collected and then analyzed by zymography. Neither cytochalasin B nor colchicine had an effect on the ConA-induced activation of proMMP-2 in HT1080 (Figure 7) and HLF1 cells (not shown) suggesting that the MT1-MMP and its putative activator(s) are not associated with the cytoskeleton.

DISCUSSION

In this study we investigated some of the cellular processes thought to play a role in the activation of proMMP-2 by ConA and TPA in two established normal (HLF1) and malignant (HT1080) fibroblast cell lines. As previously reported with other cells (7,8,16), ConA is an effective inducer of proMMP-2 activation in both cell lines generating the 62- and 59-kDa active forms. In contrast to ConA, TPA-mediated activation is cell type specific since only HT1080 cells responded. A lack of activation after TPA treatment in normal cells was also reported in primary human gingival fibroblasts (15) and in WI-38 fibroblasts (8) both of which respond to ConA. However, other study demonstrated that embryonic lung fibroblasts (CCL-137) do not respond to ConA (21). The activation of proMMP-2 induced by TPA and ConA has been shown to be mediated by MT1-MMP, a recently discovered MMP that is bound to the plasma membrane (10,11). In this study, we examined the effects of ConA and TPA on MT1-MMP expression and found a complex relationship between MT1-MMP expression and proMMP-2 activation. Although TPA caused a 2-fold increase in MT1-MMP mRNA levels in both cell types, only in HT1080 cells proMMP-2 was activated after TPA treatment, suggesting that in HLF1 cells induction of MT1-MMP expression alone is not sufficient to induce proMMP-2 activation. This was not the case after ConA treatment where we found a positive correlation between induction of MT1-MMP expression and proMMP-2 activation in both cell types as previously shown with a breast cancer cell line (16). Consistently, addition of mannose, the competitive sugar, inhibited ConA-mediated proMMP-2 activation and MT1-MMP mRNA expression. Thus, in HLF1 and HT1080 cells, transcriptional regulation of MT1-MMP expression is a necessary step in the cascade of events leading to proMMP-2 activation. However, in spite of the expression of MT1-MMP mRNA, untreated HLF1 cells did not show presence of detectable amounts of MT1-MMP protein and consequently did not activate proMMP-2. In untreated HT1080 cells, expression of MT1-MMP protein was not sufficient to cause proMMP-2 activation suggesting that the activation of proMMP-2 requires both active

transcription of MT1-MMP-1 mRNA expression and another yet unidentified element(s) that, depending on the cell type and signal, may include synthesis and processing of MT1-MMP protein into an active membrane-bound enzyme or production of a co-factor as suggested by Yu et al (16) for breast cancer cells. A recent study reported that induction of MT1-MMP mRNA expression in an embryonic lung fibroblast cell line by TPA or ConA did not result in activation of proMMP-2 consistent with the need of additional co-operating factor(s) for this process (21). It is possible, therefore, that the differential response to ConA, in terms of proMMP-2 activation, between fibroblast cell lines (21), in spite of the similar induction of MT1-MMP expression, may be related to the presence or absence of this additional co-factor(s).

To define the signaling pathway leading to proMMP-2 activation after exposure to lectins we compared ConA with succinylated ConA and found that proMMP-2 activation did not take place when either cell type was treated with succinylated ConA even at high concentrations. Since these two variants possess the same carbohydrate specificity, differences in response may be related to the dimeric conformation of succinyl ConA as compared to the tetramer configuration of ConA. The latter facilitates the clustering of glycosylated ligands on the cell surface that may initiate activation of intracellular signalling pathways. The carbohydrate requirement for the induction of proMMP-2 activation was examined using ConA and WGA. We have found that both lectins were equally active in HLF1 and HT1080 cells suggesting that GlcNAc, mannose and sialic acid oligosaccharide-containing glycoprotein(s) may all be involved in this process. Consistently, the specific competitive sugars, i.e., mannose and GlcNAc but not galactose or GalNAc inhibited proMMP-2 activation demonstrating that the effect of ConA is mediated by carbohydrate recognition. However, the WGA effect was inhibited only by the presence of GlcNAc suggesting that the WGA-induced activation of proMMP-2 is mediated by its binding to a GlcNAc-containing cell surface molecule. The relevance of the carbohydrate moieties was further demonstrated by the ability of mannose to specifically inhibit the induction of MT1-MMP mRNA expression by ConA. In agreement with this result, a previous study showed that induction of MMP-2 mRNA by ConA in human gingival fibroblasts was inhibited by α -methyl-D-mannopyranoside (15) suggesting that activation is not limited to the plasma membrane compartment but involves a signal transduction pathway from the cell surface to the nucleus.

While ConA clearly induces proMMP-2 activation in fibroblast cells, it also causes significant changes in cell morphology with cells adopting a rounded shape compared to the classical elongated spindle shape of untreated fibroblasts (15). Thus, ConA treatment may affect the organization of the cytoskeleton. Another study showed that proMMP-2 activation in human breast cancer cell lines could be induced by culturing the cells on a 3-dimensional gel of collagen I (9). This substrate is well known for its ability to influence many cellular activities involving changes in cell shape including adhesion, spreading, contraction, and cell motility. Since ConA and TPA treatment may induce translocation of MT1-MMP protein to the plasma membrane, it was noteworthy to find that both cytochalasin B and colchicine, two agents known to disrupt microfilaments and microtubules, respectively, had no effect on proMMP-2 activation in HLF1 and HT1080 cells. This lack of effect is also intriguing given the fact that succinyl-ConA, which does not induce receptor clustering, was inactive in promoting proMMP-2 activation. These results suggest that translocation and localization of MT1-MMP to the plasma membrane may not be dependent on the organization of the cytoskeleton. Likewise, the intracellular signalling pathway activated by ConA and TPA remains functional under conditions that disrupt the organization of the cytoskeleton. In summary, the results presented here show that normal and tumorigenic human fibroblasts respond differently to inducers of proMMP-2 activation

and that activation involves a signal transduction pathway of carbohydrate recognition and clustering ligand(s) and the action of a yet uncharacterized factor.

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Expression of functional recombinant human procathepsin B in mammalian cells

Wei-Ping REN*, Rafael FRIDMAN†, James R. ZABRECKY‡, Leticia D. MORRIS‡, Nancy A. DAY* and Bonnie F. SLOANE*§

Departments of *Pharmacology and †Pathology, Wayne State University, Detroit, MI 48201, and ‡Oncogene Science, Cambridge, MA 02412, U.S.A.

Cathepsin B has been implicated in numerous pathobiological processes. In order to study its interactions with other proteins implicated in these processes, quantities of functional recombinant cathepsin B are needed. Therefore, we expressed recombinant human procathepsin B in mammalian cells (BSC-1 monkey kidney cells and HeLa human cervical carcinoma cells) using a vaccinia virus expression system. The recombinant human procathepsin B appeared to be authentic and expressed in its native conformation as indicated by: (1) N-terminal sequencing; (2) molecular size; (3) processing intracellularly to mature double-chain cathepsin B; (4) *in vitro* cleavage by pepsin to mature

cathepsin B coincident with appearance of activity against a selective synthetic substrate; and (5) substrate/inhibitor profiles. This is the first report of the expression of functional recombinant human procathepsin B in mammalian cells. We also report a single-step immunoaffinity purification procedure for the isolation of electrophoretically pure proenzyme. By the methodologies described, human procathepsin B can now be obtained in high yield. This should facilitate studies of its interactions with protease inhibitors, other proteases, extracellular matrices, cell-surface proteins and biological substrates that may be of relevance to the pathobiological functions of this enzyme.

INTRODUCTION

The lysosomal cysteine protease cathepsin B has been implicated in many pathobiological processes. Among these are tumour progression (for review, see [1]), hormone-induced tissue regression and apoptosis [2], myelocyte differentiation/macrophage activation [3,4], Alzheimer's disease [5], arthritis [6], antigen processing [7], cartilage degradation [8] and osteoclastic bone resorption [9]. In cancers, there are increases in the expression of cathepsin B at the mRNA, protein and activity levels as well as alterations in trafficking, localization and secretion of this enzyme (for a review, see [1]). The 5'-untranslated and translated regions of the cathepsin B gene are alternatively spliced, potentially altering the molecular forms expressed and the levels of expression [10,11]. Although transcriptional mechanisms for altering the expression of cathepsin B have not been identified, increased expression of cathepsin B in human tumours has been associated with malignant progression and/or poor prognosis (for a review, see [1]). Nevertheless, we know little about how either procathepsin B or mature cathepsin B might function in malignant progression or in other pathobiological processes.

We were interested in expressing functional human procathepsin B in amounts sufficient to facilitate study of interactions of this enzyme with protease inhibitors, other proteases, extracellular matrices, cell-surface proteins and biological substrates. Functional procathepsin B has not been produced with *Escherichia coli* expression systems [12,13] although Kuhelj et al. [13] have recently reported successful renaturation. Activatable forms of both rat and human procathepsin B have been produced with a yeast expression system [14,15]. The yeast expression system, however, has limitations as the procathepsin B is expressed as an α -factor fusion protein and is heterogeneously glycosylated. This

heterogenous glycosylation has been reported to reduce enzymic activity [14].

Vaccinia virus expression systems have proved useful for high levels of expression of genes in mammalian cells [16]. Vaccinia virus expression systems correctly synthesize and process proteins and can be used to study trafficking and localization of proteins, e.g. *ras* and *ras*-related proteins [17]. We report here the first production of functional human procathepsin B in mammalian cells using a vaccinia virus expression system. In addition, we report the development of a single-step immunoaffinity method for purification of human procathepsin B.

EXPERIMENTAL

Materials

The following cell lines were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.): BSC-1 monkey kidney cells, HeLaS3 human cervical carcinoma cells, CV-1 African green monkey kidney cells and TK-143B, a human sarcoma cell line lacking the thymidine kinase (TK) gene. The substrates benzyloxycarbonylarginylarginyl-7-amido-4-methylcoumarin (Z-Arg-Arg-NHMec), benzyloxycarbonylphenylalanyl-arginyl-7-amido-4-methylcoumarin (Z-Phe-Arg-NHMec) and L-arginyl-7-amido-4-methylcoumarin (L-Arg-NHMec) were purchased from Enzyme Systems Products (Livermore, CA, U.S.A.); *Nco*I and *Bam*HI restriction enzymes, Lipofectin, Opti-MEM and minimal essential medium (MEM) from GIBCO-BRL (Grand Island, NY, U.S.A.); methionine-free MEM from Quality Biological (Gaithersburg, MD, U.S.A.); [³⁵S]methionine (800–1000 Ci/mmol) from DuPont-New England Nuclear (Boston, MA, U.S.A.); peptide-N²-(N-acetyl- β -glucosaminyl)-asparagine amidase (PNGase F) and endoglycosidase H (endo H) from Boehringer-Mannheim (Indianapolis, IN, U.S.A.);

Abbreviations used: BUdR, 5-bromo-2'-deoxyuridine; E-64, 1-L-*trans*-epoxysuccinyl-leucylamido-3-(4-guanidino)butane; endo F or H, endoglycosidase F or H; L-Arg-NHMec, L-arginyl-7-amido-4-methylcoumarin; MEM, minimal essential medium; NP-40, Nonidet P-40; p.f.u., plaque-forming units; PNGase F, peptide-N²-(N-acetyl- β -glucosaminyl)-asparagine amidase; TK, thymidine kinase; Z-Arg-Arg-NHMec, benzyloxycarbonylarginylarginyl-7-amido-4-methylcoumarin; Z-Phe-Arg-NHMec, benzyloxycarbonylphenylalanylarginyl-7-amido-4-methylcoumarin.

§ To whom correspondence and reprint requests should be addressed.

tunicamycin from Fluka (Ronkonkoma, NY, U.S.A.); aprotinin, Brij, 5-bromo-2'-deoxyuridine (BUdR), 3-(cyclohexylamino)-1-propanesulphonic acid, 1-L-*trans*-epoxysuccinyl-leucylamido-3-(4-guanidino)butane (E-64), Mops, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid, 2-(*N*-morpholino)ethanesulphonic acid, Nonidet P-40 (NP-40), pepstatin A, porcine pepsin, PMSF, Ponceau S, Triton X-100 and Tween from Sigma (St. Louis, MO, U.S.A.); Protein A-Sepharose, pI markers and precast isoelectric focusing gels from Pharmacia LKB (Piscataway, NJ, U.S.A.); Affigel-10, nitrocellulose membranes and horseradish peroxidase-conjugated goat anti-(rabbit IgG) from Bio-Rad (Melville, NY, U.S.A.); precast tricine gels, tricine/SDS running buffer and poly(vinylidene difluoride) membranes from Novex (San Diego, CA, U.S.A.); the enhanced chemiluminescence Western blot detection system from Amersham (Arlington Heights, IL, U.S.A.); primers for PCR from Operon (Alameda, CA, U.S.A.); and the Wizard DNA Clean-Up system from Promega (Madison, WI, U.S.A.). The source of molecular mass markers depended on the technique being used: for fluorography, ^{14}C -methylated ovalbumin and carbonic anhydrase were obtained from DuPont-New England Nuclear; for SDS/PAGE, Dalton VII-L were from Sigma; and for immunoblotting, low-range biotinylated SDS/PAGE standards were from Bio-Rad and rainbow markers from Amersham. The monoclonal antibody (DC1) to a propeptide sequence from human procatepsin B was developed by Oncogene Science (Uniondale, NY, U.S.A.). The purification of human liver cathepsin B [18] and the characterization of the rabbit anti-(human liver cathepsin B) serum and IgG [18] and the rabbit anti-(human cathepsin B propeptide) IgG [19] have been described previously. All other chemicals were of reagent grade and were obtained from commercial sources.

Cloning of cDNA and construction of expression vector for human preprocatepsin B

The full-length coding sequence of human preprocatepsin B was amplified by PCR using a human preprocatepsin B cDNA isolated from a gastric adenocarcinoma cDNA library [20] as the template. A 5' oligonucleotide [5'-GGC GCCATGGCT TGG CAG CTC TGG with a recognition sequence for *Nco*I (underlined)], a 3' oligonucleotide [5'-CGGGATCCT TAG ATC TTT TCC CAG TA with a recognition sequence for *Bam*HI (underlined)] and a termination codon were designed based on our published human cDNA sequence [20]. The upstream primer inserts an extra alanine codon (GCT) immediately after the ATG initiation codon. The DNA was amplified in a Perkin-Elmer Geneamp 9600 DNA Thermal Cycler by a PCR cycle (95 °C for 30 s, 58 °C for 30 s, 75 °C for 1 min) repeated 30 times. The amplified full-length cDNA fragment of cathepsin B was digested with *Nco*I and *Bam*HI, purified with the Wizard DNA Clean-Up system and ligated into purified pTF7-EMCV-1 [21] that had been digested with *Nco*I/*Bam*HI. The resulting plasmid containing the cDNA for human preprocatepsin B under the control of the T7 promoter was designated pTF7CB3. The PCR-generated cDNA fragment was sequenced by the dideoxy method [22] to ensure that it was in frame and without any amino acid substitutions.

Vaccinia virus expression of human preprocatepsin B

Virus infection/plasmid transfection

BSC-1 cells (2×10^6 /35-mm-diam. dish) were infected with vTF7-3, a recombinant vaccinia virus containing the bacteriophage T7 RNA polymerase gene [23], at a multiplicity of 30 plaque-forming units (p.f.u.) per cell. The virus was allowed to adsorb

for 30 min and the inoculum replaced with 1 ml of Opti-MEM. The infected BSC-1 cells were then transfected with 10 µg of plasmid pTF7CB3 per dish using Lipofectin according to the directions of the manufacturer.

Isolation of recombinant vaccinia virus containing the human cathepsin B gene

CV-1 cells (2×10^6 /35-mm-diam. dish) were infected with 0.05 p.f.u./cell of wild-type vaccinia virus (strain WR) and transfected with 20 µg of plasmid pTF7CB3 as described above. Forty-eight hours after infection/transfection, the cells were harvested and a crude stock of vaccinia virus was prepared as described [24]. Recombinant viruses containing human preprocatepsin B, designated vTF7CB3, were selected by plaque assay on TK⁻143B cell monolayers in the presence of BUdR (25 µg/ml). TK⁻ recombinant virus plaques were distinguished from spontaneous TK⁻ mutant viruses by DNA dotblot hybridization. After three consecutive plaque purifications, the recombinant viruses were amplified by infecting TK⁻143B cell monolayers in the presence of BUdR, and then were propagated on HeLa cells without selection.

Virus co-infection

Suspension cultures of HeLa cells (7×10^6) were co-infected with a mixture of vTF7-3 and vTF7CB3 at a multiplicity of 5 p.f.u. per cell for each virus. After infection, the cells were cultured for 2 days in Opti-MEM. The media were collected and clarified by centrifugation (28000 g for 120 min at 4 °C). The supernatant was concentrated 10-fold in an Amicon model 8200 concentrator fitted with a YM 10 membrane (Danvers, MA, U.S.A.) and subjected to immunoaffinity chromatography as described below or subjected to SDS/PAGE, transferred to nitrocellulose membranes and analysed by immunoblotting with a rabbit anti-(human liver cathepsin B) IgG.

Isolation of recombinant human procatepsin B by immunoaffinity chromatography

Using a monoclonal antibody generated to a propeptide sequence of human procatepsin B (DC1), an immunoaffinity column was prepared by coupling the monoclonal antibody to Affi-Gel 10 (particle size 70–300 µm) overnight at 4 °C with gentle rotation in the presence of 100 mM Mops, pH 7.5. All subsequent steps were carried out at 4 °C. The buffer was removed by centrifugation at 1000 g for 2 min and the active ester residue blocked by incubation with 1 M ethanolamine, pH 8.0 (0.1 ml/ml of gel), for 1 h with rotation. The gel was then packed in a small column (1.6 cm × 20 cm) and equilibrated with loading buffer [10 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulphonic acid (pH 7.2), 150 mM NaCl, 10% (w/v) glycerol and 0.5 mM PMSF] at a flow rate of 25 ml/h. Concentrated medium from co-infected HeLa cells was applied over the affinity resin and recycled a second time. The unbound fraction was collected and the column washed with loading buffer at a flow rate of 0.5 ml/min until the A_{280} returned to zero. Bound procatepsin B was eluted with 100 mM glycine, pH 3.0, containing 0.5 mM PMSF. Twenty fractions of 1 ml were collected and neutralized to pH 7.4 with 1 M phosphate buffer, pH 8.5; the fractions were pooled and concentrated to a volume of 1–2 ml as described above and stored at –80 °C. The purity of the recombinant procatepsin B was confirmed by SDS/PAGE under reducing conditions followed by silver staining according to the method of Merrill et al. [25].

Characterization of recombinant human procathepsin B

Metabolic labelling and immunoprecipitation

Four to five hours after transfecting BSC-1 cells (2×10^6 /6-cm-diam. dish) with pTF7CB3 or co-infecting HeLa cells (2×10^6 /35-mm-diam. dish) with a mixture of vTF7-3 and vTF7CB3 (at a multiplicity of 5 p.f.u. per cell for each virus), methionine-free MEM containing dialysed 1% (v/v) fetal bovine serum was substituted for Opti-MEM for 15 min to starve the cells of methionine. The cells were then metabolically labelled with 50 μ Ci/ml [35 S]methionine for 18 h. Media were collected and clarified by centrifugation at 1000 g for 10 min. Cells were harvested with a lysis buffer [0.5% SDS, 50 mM Tris (pH 9.0), 100 mM NaCl and 2 mM EDTA], boiled for 3 min and the supernatant collected by centrifugation at 7800 g for 10 min at 4 °C. Media and cell lysates (50 μ l) were diluted to a final volume of 250 μ l with immunoprecipitation buffer [50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.05% Brij, 1 mM PMSF and 10 μ g/ml aprotinin] and incubated with 20 μ l of rabbit anti-(human liver cathepsin B) serum at 4 °C overnight. Antigen-antibody complexes were bound to Protein A-Sepharose, eluted with sample loading buffer (0.1 M Tris buffer, pH 6.8, containing 3% SDS, 20% glycerol and 0.002% Bromophenol Blue) and after boiling for 3 min were analysed by SDS/PAGE under reducing conditions and visualized by fluorography.

Immunoblotting

Media and cell lysates of HeLa cells co-infected with both vTF7-3 and vTF7CB3 were prepared as described above. Samples were electrophoresed on SDS/polyacrylamide gels under reducing conditions and transferred to nitrocellulose membranes. The membranes were developed with an enhanced chemiluminescence Western blot detection system using reconstituted dried milk (10%) and Tween as blocking agents, rabbit IgG raised against human liver cathepsin B or against a synthetic peptide derived from the N-terminus of the propeptide of human cathepsin B as the primary antibody, and horseradish peroxidase-conjugated goat anti-(rabbit IgG) as the secondary antibody [18,19].

Isoelectric focusing

Immunoaffinity-purified recombinant human procathepsin B (200 ng) and purified human liver cathepsin B (200 ng) were loaded on precast isoelectric focusing gels (pI range 3.0–9.0) and subjected to isoelectric focusing and silver staining according to the protocol for the Phast System (Pharmacia-LKB).

N-terminus sequence analysis

Immunoaffinity-purified recombinant human procathepsin B was run on 16% tricine/SDS/polyacrylamide gels, electrophoretically transferred to a poly(vinylidene difluoride) membrane (0.2 μ m pore size) using 10 mM 3-(cyclohexylamino)-1-propanesulphonic acid, pH 11.0, and stained with Ponceau S followed by a methanol/water destaining. Direct protein microsequencing from the poly(vinylidene difluoride) membrane was performed by Dr. Jan Pohl, Director of the Microchemical Facility of the Winship Cancer Center of the Emory University School of Medicine (Atlanta, GA, U.S.A.).

Pepsin processing

Media of HeLa cells containing recombinant human procathepsin B were incubated with pepsin (4 μ g/ml) in 50 mM sodium formate buffer, pH 3.0, at 37 °C for time periods from 0 to

90 min and analysed for activity against Z-Arg-Arg-NHMec as described below. Immunoaffinity-purified recombinant human procathepsin B (1 μ g) was incubated with pepsin (0.002 or 0.01 μ g) as described for media. Processing was analysed by separation of molecular forms on SDS/PAGE under reducing conditions, transfer to nitrocellulose membranes and immunoblotting as described above.

Analysis of glycosylation

To inhibit N-linked glycosylation during biosynthesis of cathepsin B, tunicamycin (2 μ g/ml) was added to infected BSC-1 cells immediately after their transfection with pTF7CB3. The cells were metabolically labelled and the molecular forms of cathepsin B immunoprecipitated and visualized by fluorography. To evaluate the types of oligosaccharides, recombinant human procathepsin B was incubated with PNGase F or endo H [18]. Immunoaffinity-purified recombinant procathepsin B (1 μ g) from HeLa cells was incubated for 16 h at 37 °C alone (pH 7.0) or with 600 m-units of PNGase F in 50 mM sodium phosphate buffer (pH 7.0), containing 5 mM EDTA and 0.5% NP40, or with 10 m-units of endo H in 50 mM sodium citrate buffer, pH 5.5. Cathepsin B was then subjected to SDS/12%-PAGE under reducing conditions followed by Coomassie Blue staining.

Determination of activity of recombinant human cathepsin B

The activity of cathepsin B in homogenates and conditioned media from BSC-1 cells was determined in triplicate against the selective substrate Z-Arg-Arg-NHMec (10 μ M final concentration) using our published protocol [26]. Enzyme activity is expressed as nmol of NHMec released/min per 10^5 cells. The presence of latent activity due to expression of procathepsin B was assessed by activation with pepsin (0.05 mg/ml) in 0.1 M formate buffer, pH 3.0, at 37 °C for 15 min [19]; latent activity is expressed as the difference between the total activity of both pro and mature forms of cathepsin B measured in the presence of pepsin and the activity of mature cathepsin B measured in the absence of pepsin. At 48 h after infection/transfection, conditioned media were collected and clarified by centrifugation at 1000 g for 10 min at 4 °C. Adherent cells were washed with PBS and harvested with a rubber policeman into prechilled (4 °C) homogenization buffer {25 mM 2-(*N*-morpholino)ethanesulphonic acid (pH 6.5), 0.25 M sucrose and 1 mM EDTA}. The cell suspension was homogenized on ice using 40 strokes of a Dounce homogenizer fitted with a type A pestle. Triton X-100 was added to the homogenate to a final concentration of 0.1%.

Active recombinant human cathepsin B was generated by incubation of immunoaffinity-purified recombinant human procathepsin B (2 μ g) with pepsin (0.01 μ g) at 37 °C for 20 min in the presence of 0.1 M formate buffer, pH 3.0, containing 1 mM EDTA. The activation reaction was stopped by adding pepstatin A at a final concentration of 10 μ M. Activities of recombinant human cathepsin B and of purified human liver cathepsin B against three substrates (Z-Arg-Arg-NHMec, Z-Phe-Arg-NHMec and L-Arg-NHMec) were then compared at a final substrate concentration of 10 μ M. Human liver cathepsin B was purified as described previously [18].

RESULTS AND DISCUSSION

Expression of recombinant human procathepsin B in mammalian cells

Human procathepsin B was expressed in BSC-1 cells by infection with a recombinant vaccinia virus containing the bacteriophage

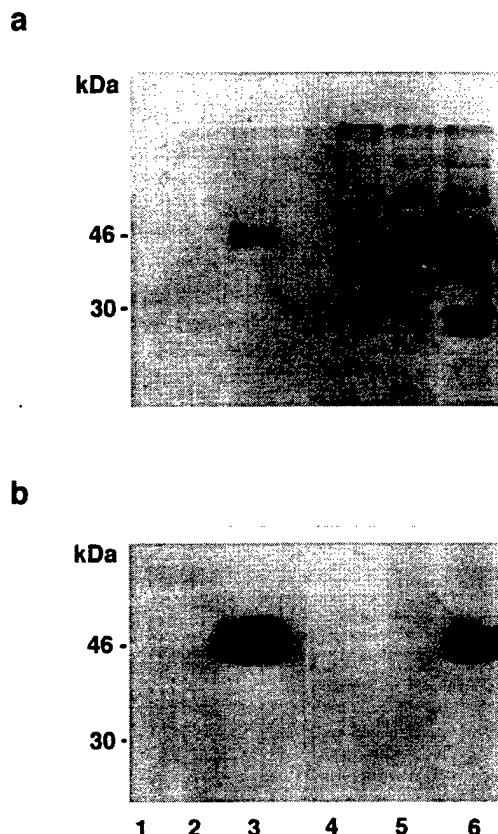


Figure 1 Expression of recombinant human procathepsin B in mammalian cells

Each lane represents 1×10^5 cells; molecular masses (kDa) are shown on the left. (a) Recombinant human procathepsin B was expressed in BSC-1 cells by infection with vTF7-3, a recombinant vaccinia virus containing the bacteriophage T7 RNA polymerase gene, and transfection with pTF7CB3, a plasmid containing a human preprocathepsin B cDNA under the control of the T7 promoter. The biosynthesis, intracellular processing and secretion of recombinant human procathepsin B was followed by metabolic labelling, immunoprecipitation, SDS/12%-PAGE and fluorography of 50 μ l of conditioned media (lanes 1–3) and cell lysates (lanes 4–6). Lanes represent: 1 and 4, control uninfected cells; 2 and 5, cells infected with vTF7-3; and 3 and 6, cells infected with vTF7-3 and transfected with pTF7CB3. (b) Recombinant human procathepsin B was expressed in HeLa cells by co-infection with vTF7-3 and vTF7CB3, a recombinant vaccinia virus containing the human preprocathepsin B gene under the control of the T7 promoter. The biosynthesis and secretion of recombinant human procathepsin B was followed by metabolic labelling, immunoprecipitation, SDS/12%-PAGE and fluorography of 50 μ l of conditioned media (lanes 1–3) and cell lysates (lanes 4–6). Lanes represent: 1 and 4, cells infected with vTF7-3 alone; 2 and 5, cells infected with vTF7CB3 alone; and 3 and 6, cells co-infected with vTF7-3 and vTF7CB3.

T7 polymerase gene (vTF7-3) and transfection with pTF7CB3, a plasmid containing a human preprocathepsin B cDNA under the control of the T7 promoter. A protein of ~ 45 kDa was immunoprecipitated from the conditioned media of the transfected cells by an anti-(human cathepsin B) serum (Figure 1a, lane 3). In the cell lysate, we identified a 26/25 kDa doublet and a 45/43 kDa doublet (Figure 1a, lane 6). These proteins were not observed, under these conditions, in immunoprecipitates of media from untransfected BSC-1 cells (Figure 1a, lanes 1 and 2) or in immunoprecipitates of lysates from untransfected BSC-1 cells (Figure 1a, lanes 4 and 5). Endogenous monkey kidney cathepsin B was not immunoprecipitated by the anti-(human cathepsin B) serum (Figure 1a, lane 4). The 45/43 kDa bands are similar in size to the two bands of procathepsin B observed in pulse-chase studies of metabolically labelled human skin fibroblasts [27]. The

26/25 kDa doublet is comparable to the glycosylated and unglycosylated heavy-chain doublet of purified human liver and sarcoma cathepsin B [18]. The presence of a 26/25 kDa doublet in the lysates of BSC-1 cells suggests that the recombinant human procathepsin B was able to undergo complete processing to the mature double-chain form and, thus, had been correctly folded.

We further characterized the recombinant procathepsin B by immunoblotting of BSC-1 cell lysates with purified IgG fractions of two rabbit anti-(human cathepsin B) polyclonal antibodies. One of the antibodies was raised against purified human liver cathepsin B and recognizes all forms of the enzyme [18,19]; the second was raised against a synthetic peptide sequence at the N-terminus of the human cathepsin B propeptide and recognizes only procathepsin B [19]. The ~ 45 kDa proteins cross-reacted with both antibodies, indicating that they were indeed procathepsin B (results not shown). A 36 kDa protein of unknown origin was immunoprecipitated from the cell lysates of infected and infected/transfected BSC-1 cells (Figure 1a, lanes 5 and 6). This protein was not immunoprecipitated from the uninfected cells (Figure 1a, lane 4), nor was it recognized by either of the anti-(cathepsin B) antibodies (results not shown).

We constructed a recombinant vaccinia virus containing the human preprocathepsin B gene (vTF7CB3) under the control of the T7 promoter and co-infected HeLa cells with this virus and vTF7-3 in order to obtain milligram quantities of procathepsin B for biochemical studies. Co-infection of HeLa cells resulted in expression of procathepsin B in the media and cell lysates as determined by the presence of a 46/43 kDa doublet after immunoprecipitation (Figure 1b, lanes 3 and 6). The 26/25 kDa doublet of mature cathepsin B was not observed in the lysates of HeLa cells. The level of expression of recombinant human procathepsin B in the HeLa cells was ~ 0.2 mg/ 10^8 cells as determined by comparison with known amounts of mature single-chain and double-chain human liver cathepsin B (results not shown). This is comparable with the level of expression of other proteins with vaccinia virus expression systems, e.g. 72 kDa progelatinase A [28]. Our results indicate that most of the recombinant procathepsin B expressed in co-infected HeLa cells was not processed to mature cathepsin B. This may reflect expression of a lysosomal protease in a cancer cell line, as cathepsin D in human breast carcinomas [29] and cathepsin L in malignantly transformed fibroblasts [30,31] are primarily secreted as pro forms with little or no enzyme processed intracellularly.

Enzymic activity of recombinant human cathepsin B

Cathepsin B activity

Z-Arg-Arg-NHMec-hydrolysing activity was measured in the conditioned media and cell homogenates of control, infected and infected/transfected BSC-1 cells (Figure 2), before and after activation by pepsin with the difference representing the latent activity (i.e. the activity due to procathepsin B). Prior to treatment with pepsin, there was little activity against the cathepsin B substrate in the media of any of the BSC-1 cells (Figure 2: Media, mature). After treatment with pepsin, there was a 25-fold increase in cathepsin B activity in the media of infected/transfected cells (Figure 2: Media, latent), i.e. in those cells shown by immunoprecipitation to secrete recombinant procathepsin B (cf. lane 3 in Figures 1a and 1b). There was little activity in the media of control and infected cells after treatment with pepsin (Figure 2: Media, latent), indicating that these cells secreted little or no endogenous procathepsin B. There was endogenous cathepsin B activity in the homogenates of control cells (Figure 2: Cells, mature), but this could not be increased by pepsin, suggesting that the endogenous enzyme was in the mature form. The activity

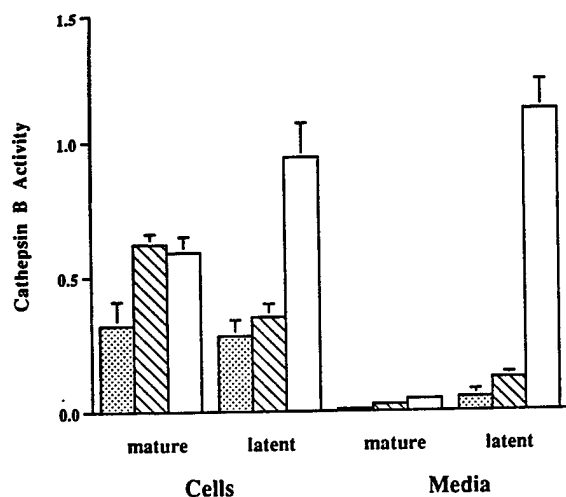


Figure 2 Analyses of cathepsin B activity in BSC-1 cells and conditioned media

Mature activity represents the activity measured against Z-Arg-Arg-NHMec prior to incubation with pepsin, whereas latent activity represents the activity due to procathepsin B (assessed as the difference in activities measured before and after incubation with pepsin). The stippled bars represent activity in control cells; the striped bars in cells infected with vTF7-3; and the open bars activity in cells infected with vTF7-3 and transfected with pTF7CB3. Activity is expressed as nmol of NHMec/min per 10⁵ cells; mean \pm S.D.

of mature recombinant cathepsin B in homogenates of infected/transfected cells was 70% greater than in homogenates of control BSC-1 cells (Figure 2: Cells, mature) and that of latent or procathepsin B in infected/transfected cells was 200% greater (Figure 2: Cells, latent). These results support the conclusion drawn from our immunoprecipitation studies that both mature and pro forms of recombinant cathepsin B were present in infected/transfected BSC-1 cells.

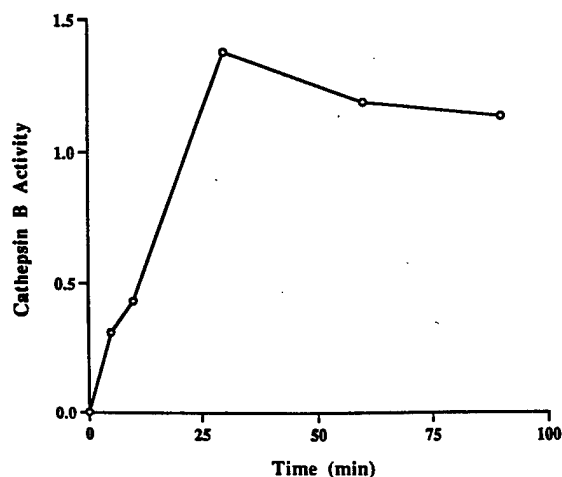


Figure 3 Activation of recombinant procathepsin B by pepsin

Concentrated (10 \times) medium (2 ml) from co-infected HeLa cells was incubated with pepsin (10 ng) at pH 3.0 for the indicated time periods at 37 $^{\circ}$ C and then assayed for activity against Z-Arg-Arg-NHMec. Activity is expressed as nmol of NHMec/min per 10⁵ cells.

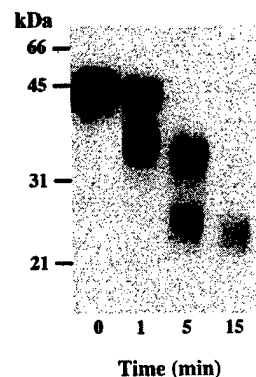


Figure 4 Molecular forms of recombinant cathepsin B generated by incubation with pepsin

Immunoaffinity-purified recombinant human procathepsin B (1 μ g) was incubated with pepsin (10 ng) at pH 3.0 and 37 $^{\circ}$ C for the time periods indicated. Samples were separated on SDS/12% polyacrylamide gels, transferred to nitrocellulose membranes and analysed by immunoblotting with a rabbit anti-(human liver cathepsin B) IgG. Molecular masses (kDa) are shown at the left.

Pepsin activation of recombinant human procathepsin B

There is some controversy in the literature as to the enzyme(s) responsible for activation of procathepsin B *in vivo* [15,32]. Nevertheless, procathepsin B secreted into the media of tumour cells can be activated *in vitro* by incubation of the media with pepsin [19,33]. Therefore, we determined whether the recombinant human procathepsin B expressed in mammalian cells with the vaccinia virus system was synthesized in a conformation that could be activated by pepsin. We demonstrate that pepsin was able to cleave recombinant procathepsin B to yield active cathepsin B and protein products of the correct size whether the substrate for pepsin was recombinant procathepsin B secreted into conditioned medium and thus in a complex mixture of other proteins (Figure 3 and results not shown), or was immunoaffinity-purified recombinant procathepsin B (Figure 4 and results not shown). In Figure 3, a time-dependent increase in cathepsin B activity is depicted; this was maximal (1.4 nmol/min per 10⁵ cells) after 30 min of incubation. When immunoaffinity-purified recombinant procathepsin B (see below) was incubated with pepsin, single-chain cathepsin B could be observed within 1 min and both single- and double-chain cathepsin B within 5 min of incubation (Figure 4). The ability of pepsin to generate cathepsin B activity and the appropriate mature forms of cathepsin B indicates that the recombinant enzyme produced with the vaccinia virus expression system was in a native conformation.

Characterization of recombinant human cathepsin B: substrate specificity and inhibition

We compared the activities of immunoaffinity-purified recombinant human cathepsin B and human liver cathepsin B against three synthetic substrates. Cathepsin B had previously been shown to have a greater k_{cat}/K_m against a Z-Phe-Arg substrate than against a Z-Arg-Arg substrate [18]. The activities of recombinant and liver cathepsin B were 40–50% greater against Z-Phe-Arg-NHMec than against Z-Arg-Arg-NHMec. Activities of both enzymes (tested against the di-Arg substrate) could be inhibited > 99% by 10 μ M E-64, an irreversible cysteine protease inhibitor often used as an active-site titrant for cathepsin B and other cysteine proteases [34]. Neither enzyme was able to cleave

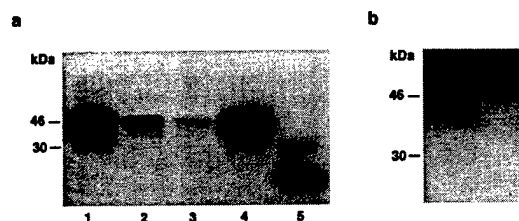


Figure 5 Reducing SDS/PAGE of recombinant human procathepsin B secreted from co-infected HeLa cells and purified by immunoaffinity chromatography

Molecular masses (kDa) are shown on the left. (a) Samples (20 μ l) from various purification steps were separated on SDS/12% polyacrylamide gels, transferred to nitrocellulose membranes and analysed by immunoblotting with a rabbit anti-(human liver cathepsin B) IgG. Lanes represent: 1, concentrated conditioned medium; 2, unbound fraction; 3, wash fraction; 4, pool of recombinant procathepsin B eluted with glycine; and 5, 25 ng of human liver cathepsin B. (b) Affinity-purified procathepsin B (200 ng) visualized by silver-staining.

the unblocked aminopeptidase substrate L-Arg-NHMec. Thus, the substrate/inhibitor profiles for recombinant human cathepsin B and human liver cathepsin B were similar, again suggesting that the recombinant cathepsin B had been correctly folded.

Immunoaffinity purification of recombinant human procathepsin B

Recombinant procathepsin B bound to an immobilized monoclonal antibody generated against a human cathepsin B pro-peptide sequence and could be eluted with 100 mM glycine at pH 3.0 (Figure 5a, lane 4). Recovery of recombinant human procathepsin B from concentrated conditioned media of HeLa cells was $\geq 95\%$ (as estimated by quantification of immunoblots) with a yield of ~ 1.0 mg of purified procathepsin B per 5×10^8 cells. Affinity-purified recombinant human procathepsin B ran as a diffuse band of ~ 45 kDa in silver-stained SDS/polyacrylamide gels under reducing conditions (Figure 5b). Our yield of purified recombinant human procathepsin B from HeLa cell medium was an order of magnitude greater (12–13-fold) than has been reported for the yield of purified recombinant human procathepsin B from yeast culture medium by Mach et al. [15]. Our yield was, however, 4–5-fold less than has been reported for that of a proenzyme mutant of rat cathepsin B produced in yeast [35]. The latter is an active-site mutant in which the active-site Cys has been mutated to Ala and thus the expressed enzyme is not functional. Furthermore, the rat and human procathepsin B expressed in yeast are α -factor fusion proteins, carrying N-terminal extensions of four amino acids [35]. We confirmed that the human procathepsin B expressed in mammalian cells was authentic procathepsin B by N-terminal sequence analysis of the first 28 amino acids (positions 18–45) of immunoaffinity-purified recombinant human procathepsin B. Twenty-four amino acids corresponded to those predicted by the cDNA [20]. The N-terminal sequence analysis was unable to distinguish between His and Ala at positions 24, 43 and 45 and predicted that position 41 was either Trp or Cys; the amino acids predicted by the cDNA for these positions were His, Ala, His and Trp, respectively [20]. The vaccinia virus expression of human procathepsin B would appear to be advantageous in that authentic human procathepsin B is expressed, the yields are high and the proenzyme can be purified to homogeneity in a single step by an immunoaffinity method. Furthermore, if one required mature forms of recombinant cathepsin B, the recombinant human procathepsin B can be activated *in vitro* with pepsin or other proteases and active cathepsin B purified by affinity ligand chromatography (results

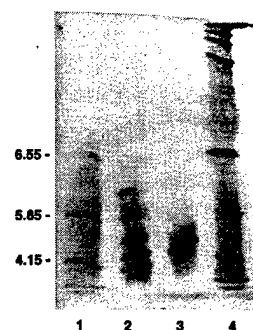


Figure 6 Isoelectric focusing of recombinant human procathepsin B and human liver cathepsin B

Samples (200 ng each) of immunoaffinity-purified recombinant human procathepsin B and human liver cathepsin B were loaded on precast isoelectric focusing gels (pI range 3.0–9.0) and silver stained. Lanes represent: 1, pI markers from 2.5 to 6.5; 2, recombinant human procathepsin B; 3, mature human liver cathepsin B; and 4, pI markers from 3 to 9.

not shown), a method used for purification of cathepsin B from a wide variety of tissues [18,36]. Thus, with the combination of vaccinia virus expression and immunoaffinity chromatography, one can obtain quantities of native human procathepsin B or cathepsin B.

Isoelectric point of recombinant human procathepsin B

Purified mature cathepsin B has previously been shown to consist of multiple isoforms. For example, crystalline rat liver cathepsin B consists of four isoforms with pIs from 4.9 to 5.3 [37]. The diffuse band observed upon silver staining of immunoaffinity-purified recombinant human procathepsin B suggested that recombinant human procathepsin B may consist of more than one isoform. The number of isoforms and their pI(s) have not been reported for recombinant procathepsin B. By isoelectric focusing, recombinant human procathepsin B was found to consist of multiple isoforms ranging from a pI of 4.0 to a pI of 6.2 (Figure 6, lane 2), whereas purified human liver cathepsin B (i.e. mature cathepsin B) consisted of one main isoform of 4.8 with other minor isoforms ranging from 4.6 to 5.2 (Figure 6, lane 3). Thus, the recombinant human procathepsin B appeared to have additional isoforms in both the more acidic and more basic range. Complicating the interpretation of these data is the finding that the isoelectric points being compared are for isoforms of mature liver cathepsin B and for isoforms of procathepsin B expressed in HeLa cells, a human cervical carcinoma line. Furthermore, the mature human liver cathepsin B was purified from tissue and presumably had high-mannose carbohydrates for targeting to the lysosomes. The recombinant human procathepsin B was secreted into conditioned media and may have acquired complex carbohydrates in the secretory pathway.

Glycosylation of recombinant human procathepsin B

Since there were multiple isoforms of recombinant human procathepsin B and there are three potential N-linked oligosaccharide sites in the human procathepsin B gene [20,38], we determined the contribution of carbohydrate to the molecular size of recombinant human procathepsin B. Procathepsin B immunoprecipitated from lysates of infected/transfected BSC-1 cells ran as a single 36 kDa band in cells labelled in the presence of tunicamycin as compared with a 45/43 kDa doublet in cells

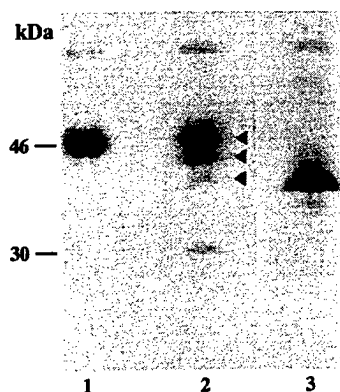


Figure 7 Analyses of glycosylation of recombinant human procathepsin B

Molecular masses (kDa) are shown on the left. Immunoaffinity-purified recombinant human procathepsin B (1 μ g) from HeLa cells was digested with endoglycosidases and subjected to SDS/12%-PAGE followed by staining with Coomassie Blue. Lanes represent: 1, control; 2, digestion with endo H; and 3, digestion with PNGase F. The arrowheads in lane 2 indicate the three bands generated by digestion with endo H.

labelled in the absence of tunicamycin (results not shown). Treatment with PNGase F, an endoglycosidase that cleaves N-linked oligosaccharides from the peptide backbone [39], also reduced the molecular size of procathepsin B from 45 to 36 kDa (results not shown and lane 3 in Figure 7). A molecular size of 36 kDa for unglycosylated or deglycosylated procathepsin B would be predicted by the cDNA sequence [20]. In cells treated with tunicamycin, no further processing of unglycosylated recombinant procathepsin B was observed over a 48 h time period. Similar observations have been made for unglycosylated procathepsin B produced in the presence of tunicamycin in human skin fibroblasts [27]. Our results thus verify that N-linked glycosylation of procathepsin B is necessary for intracellular processing, presumably because high-mannose oligosaccharides are required for generation of the phosphomannosyl recognition marker that binds to mannose-6-phosphate receptors for transport to the lysosomes [40].

Recombinant human procathepsin B might be glycosylated on one, two or all three of the putative N-linked glycosylation sites [20,38]. In addition, the nature of the oligosaccharides on any one site may differ from that on another site. Although cathepsin B as a lysosomal enzyme would be expected to have high-mannose oligosaccharides [40], Pagano et al. [38] have reported that tumour procathepsin B secreted into ascites fluid has complex oligosaccharides. To explore this, we analysed the nature of the oligosaccharides on recombinant human procathepsin B affinity-purified from the media of co-infected HeLa cells (Figure 7). Incubation of the recombinant enzyme with endo H, an endoglycosidase that cleaves high-mannose-type oligosaccharides between the two proximal GlcNAc residues of asparagine-linked carbohydrate chains [41] resulted in three molecular mass forms: an undigested form of 45 kDa, an intermediate form of 39 kDa and a deglycosylated form of 36 kDa (Figure 7, lane 2). This suggests that the procathepsin B secreted from HeLa cells was heterogeneously glycosylated: one form with high-mannose-type oligosaccharides, one form with high-mannose-type oligosaccharides on some of the N-linked glycosylation sites, and one form without high-mannose-type oligosaccharides. Pulse-chase studies of the biosynthesis and processing of cathepsin B in normal and *ras*-transformed human breast epithelial cell lines also suggest that procathepsin B

acquires both high-mannose and complex oligosaccharides (M. Sameni and B. F. Sloane, unpublished work). Similar findings have been reported for cathepsin D (e.g. [42,43]), possibly reflecting pathways alternative to the mannose phosphate receptor pathway for targeting of lysosomal proteases to the lysosome [43,44].

Recombinant procathepsin B and pathobiological function of this enzyme

The literature implicating cathepsin B in the pathology of human diseases is growing and thus there is a need to explore the interactions between pro and mature forms of cathepsin B and other molecules that play functional roles in these disease states. Immunofluorescence studies of breast cancer cell lines and *ras*-transformed breast epithelial cells have revealed cathepsin B staining on the surface of these cells [19,45], suggesting that there may be a cell-surface binding protein for cathepsin B. Access to recombinant procathepsin B will facilitate analysis of cell-surface binding. Recombinant procathepsin B will also be useful as an antigen for production and characterization of antibodies suitable for analysis of cathepsin B in tissue samples and biological fluids. Since alternative splicing of the 5' region of the cathepsin B gene can produce a truncated procathepsin B that may be specific to tumours [10], antibodies specific to the truncated propeptide might serve as tumour markers. The studies herein report for the first time the expression of functional authentic human procathepsin B in mammalian cells and in addition report a new method for immunoaffinity purification of procathepsin B. Both should contribute to biochemical studies of procathepsin B and to the production of antibodies with specificities for various molecular forms of cathepsin B.

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Phorbol Ester-induced Cell Surface Association of Matrix Metalloproteinase-9 in Human MCF10A Breast Epithelial Cells¹

Marta Toth, David C. Gervasi, and Rafael Fridman²

Department of Pathology and Karmanos Cancer Institute, Wayne State University, Detroit, Michigan 48201

ABSTRACT

Cell surface association of extracellular matrix (ECM)-degrading enzymes has been suggested to facilitate proteolysis of ECM in areas of cell-matrix contacts and to be crucial for the process of tumor cell invasion. Matrix metalloproteinase-9 (MMP-9) is a member of the MMP family of endopeptidases that has been shown to play a critical role in hydrolysis of ECM components and has been localized on the surface of tumor cells. However, the nature of the cell surface association of MMP-9 is unknown. Here, we report the cell surface association of MMP-9 in human breast epithelial MCF10A cells treated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Surface biotinylation and immunoprecipitation with anti-MMP-9 antibodies revealed the presence of two MMP-9 forms (M_r 92,000 and 85,000) on the surface of TPA-treated MCF10A cells, whereas in the media, only the M_r 92,000 form was detected, mostly in complex with TIMP-1, a specific MMP-9 inhibitor. The MMP-9 forms were also found in purified plasma membranes of TPA-treated cells. In contrast, the plasma membranes contained little or no TIMP-1. The surface-bound MMP-9 forms were recognized by an antibody to the NH₂-terminal prodomain, indicating that both represent latent enzymes. Pulse-chase analysis and endoglycosidase H digestion of surface-biotinylated MMP-9 forms demonstrated that the M_r 85,000 species was endoglycosidase H sensitive, suggesting targeting of the precursor form of MMP-9 to the cell surface. These studies demonstrate a specific cell surface association of MMP-9 in response to TPA that may help to localize TIMP-1-free enzyme on the surface of breast epithelial cells.

INTRODUCTION

The turnover of ECM³ occurs primarily at areas of cell-matrix contacts and is carried out by proteinases intimately intertwined with the cell surface. Several families of ECM-degrading proteinases have been shown to be associated with the cell surface, including MMPs (1-3), plasminogen activator (4), and cathepsins B and D (5). Cell surface association of ECM-degrading enzymes allows for localized and controlled proteolysis of ECM components and prevents complete matrix dissolution, which would otherwise adversely influence the adhesive and migratory activities of cells and lead to extensive and irreparable tissue damage. It also allows cells to strictly regulate the initiation, inhibition, and termination of proteolytic activity by concentrating specific receptors, enzymes, and inhibitors in discrete areas of the cell surface. The regulation and function of cell surface-bound proteinases and their importance for localized degradation of ECM have been very well demonstrated with the plasminogen/plasmin system, in which cells developed a complex molecular system of proteases, protease receptors, and specific inhibitors that interact with each other to regulate proteolytic activity on the cell surface (4). In

recent years, new studies provided evidence of the cell surface association and regulation of several members of the MMP family, including gelatinases (6-11) and membrane-type MMPs (3). The gelatinases, MMP-2 and MMP-9, have been shown to play a central role in the degradation of ECM during tissue remodeling, angiogenesis, arthritis, and tumor metastasis, and their expression is elevated in many human cancers (12, 13). The early studies of Zucker *et al.* (14-17) provided the first evidence of MMP-2 and MMP-9 activities on the PMs of tumor cells. Later studies showed the association of MMP-2 with the surface of transformed cells (8, 18). We have shown a pericellular localization of both MMP-2 and MMP-9 in the epithelium of fibrocystic disease of the breast and in the carcinoma cells of breast tumors (19). Others have shown the localization of MMP-9 at areas of tumor-basement membrane contacts in skin tumors (20). The association of the gelatinases with the cell surface may be an important factor in the regulation of proteolytic activity and may allow these enzymes to specifically degrade ECM proteins at areas of cell-matrix contacts and also to target surface proteins as shown with β -amyloid (21, 22), galectin-3 (23), and fibroblast growth factor receptor-1 (24).

Much information has been recently gained on the cell surface association of MMP-2, yet little is known about the interaction of MMP-9 with the cell surface. MMP-9 is produced by a variety of normal and tumor cells, including mesenchymal (25-27), epithelial (28, 29), endothelial (30), and inflammatory cells (25), and has been shown to play a role in many physiological and pathological processes, including trophoblast implantation (31), inflammation (32), bone resorption (33), arthritis (34), and cancer metastasis (35). Structurally, MMP-9 bears significant sequence similarities with MMP-2 but it also differs in substantial structural and regulatory elements (1, 12, 13). In addition to the classical domains of other MMP family members, both MMP-9 and MMP-2 contain a gelatin-binding domain that is homologous to the type II module present in fibronectin (1, 12, 13). MMP-9 possesses an additional 54-amino acid proline-rich region that is similar to the $\alpha 2(V)$ chain of collagen V that is absent from MMP-2 (25), the function of which is unknown. MMP-9, unlike MMP-2, is heavily glycosylated due to the presence of three potential *N*-linked glycosylation sites (Asn₁₉, Asn₁₀₁, and Asn₁₀₈; MMP-9₆₈₈ notation) and several less defined *O*-linked glycosylation sites (25), but the role of the oligosaccharide moieties on MMP-9 function remains unknown. Like latent MMP-2 with TIMP-2, latent MMP-9 forms a noncovalent complex with TIMP-1 (25). The binding of TIMP-1 is mediated by the COOH-terminal domain of the proenzyme form and alters enzyme activation (36-39).

In contrast to MMP-2, which is usually constitutively expressed in many cultured cells, MMP-9 expression can be induced by TPA (40), growth factors (41, 42), cytokines (43), and by yet undefined factors in *in vitro* models of tumor-stroma interactions (35, 44). In many instances, as is the case after TPA treatment, induction of MMP-9 expression and secretion is accompanied by the simultaneous induction and secretion of TIMP-1, which binds to the secreted proenzyme form (25, 40). However, TPA treatment does not result in MMP-9 activation (7, 9, 25, 40, 45). In contrast, exposure to TPA induces the activation of latent MMP-2 by MT1-MMP (7, 9). While investigating the PM-dependent activation of MMP-2, we tested PMs isolated from a nonmalignant immortalized breast epithelial cell line, MCF10A (46)

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² To whom requests for reprints should be addressed, at the Department of Pathology, Wayne State University, 540 East Canfield Avenue, Detroit, MI 48201. Phone: (313) 577-1218; Fax: (313) 577-8180; E-mail: rfridman@med.wayne.edu.

³ The abbreviations used are: ECM, extracellular matrix; MMP, matrix metalloproteinase; PMSF, phenylmethylsulfonyl fluoride; APMA, *p*-aminophenyl-mercuric acetate; ECL, enhanced chemiluminescence; HRP, horseradish peroxidase; endo-H, endoglycosidase H; PM, plasma membrane; PMCA, PM Ca²⁺ ATPase; TBS, Tris-buffered saline.

and found MMP-9 after exposing the cells to TPA. Here, we examined in detail the cell surface association of MMP-9 in MCF10A cells after TPA treatment.

MATERIALS AND METHODS

Cells. Human immortalized MCF10A breast epithelial cells, originally isolated from a patient with fibrocystic disease of the breast (46), were kindly provided by Dr. Bonnie Sloane (Wayne State University, Detroit, MI). The cells were grown in DMEM/F-12 (1:1) medium supplemented with 5% horse serum, 10 μ g/ml insulin, 100 IU/ml penicillin, 100 μ g/ml streptomycin, 0.25 μ g/ml Fungizone, 0.5 μ g/ml hydrocortisone, and 20 ng/ml epidermal growth factor. All tissue culture reagents were purchased from Life Technologies, Inc. (Grand Island, NY).

Reagents and Antibodies. TPA, aprotinin, leupeptin, pepstatin A, PMSF, APMA, tRNA, Triton X-114, and gelatin-agarose beads were all purchased from Sigma Chemical Co. (St. Louis, MO). Sulfo-NHS-biotin and the BCA protein determination kit were purchased from Pierce Chemical Co. (Rockford, IL). The ECL detection system, HRP-conjugated secondary antibodies, streptavidin-HRP, and [14 C]-labeled molecular weight standards were purchased from Amersham Corp. (Arlington Heights, IL). Percoll and Fast-flow protein G-Sepharose beads were purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Label Express [35 S]methionine was purchased from NEN, (Wilmington, DE). Endo-H (25 units/ml) was purchased from Glyko (Novato, CA). BA-S 85 nitrocellulose membrane was purchased from Schleicher & Schuell (Keene, NH). Monoclonal antibodies to TIMP-1 (Ab-1) were purchased from Calbiochem Oncogene Research Products (Cambridge, MA). An anti-MMP-9 rabbit polyclonal antibody (pAB109) raised against a synthetic peptide (APRQRQSTLVTPGDRLT) from the NH₂-terminal domain of human latent MMP-9 was a generous gift from Dr. Stetler-Stevenson (National Cancer Institute, NIH, Bethesda, MD). The production and characterization of the monoclonal antibody CA-209 against human MMP-9 and T2-101 against human TIMP-2 were described previously (19). A rabbit polyclonal antibody to human TIMP-1 was a generous gift from Dr. B. Chua (Wayne State University). A rabbit polyclonal antibody raised against a synthetic peptide (RFNEELRAVDSEYPNIK) derived from the amino acid sequence of human MT1-MMP (3, 47) was produced by and purchased from Genetics Research, Inc. (Huntsville, AL). Monoclonal antibodies to PMCA were purchased from Affinity Bioreagents, Inc. (Golden, CO).

Expression, Purification, and Activation of Human Recombinant MMP-9. Human recombinant MMP-9 was produced in a recombinant vaccinia-mammalian cell expression system and purified to homogeneity by gelatin-agarose chromatography, as described previously (48). The protein concentration of MMP-9 was determined by its molar extinction coefficient of 114,360 M⁻¹cm⁻¹ (49). To obtain a mixture of latent (M_r 92,000) and active (M_r 82,000) MMP-9 forms, the latent MMP-9 was partially activated with 1 mM APMA for 2 h at 37°C (49). To isolate the M_r 82,000 species, 10 μ g of latent MMP-9 were incubated with 200 ng of a recombinant catalytic domain of stromelysin 1 (a gift from Dr. Paul Cannon) for 2 h at 37°C (50) and then subjected to gelatin-agarose chromatography to remove stromelysin 1.

Gelatin Zymography. Gelatin zymography of conditioned media and PM fractions was performed using 10% Tris-glycine SDS-polyacrylamide gels containing 0.1% gelatin (45). Briefly, samples were mixed with Laemmli sample buffer without reducing agents and without heating and then subjected to SDS-PAGE. The gels were then incubated (30 min at 22°C) in renaturing buffer (2.5% Triton X-100 in H₂O), rinsed in distilled H₂O, and equilibrated for an additional 30 min in developing buffer (50 mM Tris buffer, pH 8.0, 200 mM NaCl, 5 mM CaCl₂, and 0.02% Brij-35) followed by an incubation (16 h at 37°C) in fresh developing buffer. The gels were then stained with 0.5% Coomassie Blue R250 in a solution of 10% methanol and 5% acetic acid and then destained in 10% methanol and 5% acetic acid.

Immunoblot Analysis. Immunoblot analyses were carried out with samples of conditioned media and purified PM fractions. Samples (200 μ l) of serum-free conditioned medium were concentrated by the addition of 20% trichloroacetic acid and 25 μ g/ml of carrier tRNA (final concentrations). The mixtures were incubated for 1 h at 4°C and centrifuged (15 min at 13,000 \times g), and the pellet was resuspended in Laemmli sample buffer and boiled. In some experiments, the serum-free conditioned medium of TPA-treated MCF10A

cells was subjected to gelatin-agarose chromatography to purify MMP-9 and the MMP-9/TIMP-1 complex as described (47). Purified PMs were resuspended in sample buffer and boiled. The samples were subjected to SDS-PAGE followed by transfer to a BA-S 85 nitrocellulose membrane. The blots were blocked overnight at 4°C with 3% BSA and 3% nonfat dry milk in 100 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.02% NaN₃ (blotto) and incubated for 1 h with the corresponding primary antibody in 20 mM Tris-HCl, pH 7.5, containing 137 mM NaCl and 0.1% Tween 20 (T-TBS). After being washed three times with T-TBS, the blots were incubated with the appropriate HRP-conjugated secondary antibody. Detection of the immune complexes was performed using the ECL system according to the manufacturer's instructions.

Cell Surface Biotinylation. MCF10A cells in 150-mm dishes were treated with 100 nM TPA (from a 2 mM stock solution in ethanol) for 2 h at 37°C. Control cells received vehicle alone (final concentration, 0.005% ethanol). After being washed four times with serum-free medium, the cells were incubated in serum free-medium for 18 h. The cells were then washed three times with cold PBS containing 0.1 mM CaCl₂ and 1 mM MgCl₂ (PBS-CM). Biotinylation of cell surface proteins was carried out by incubating the cells with 0.5 mg/ml of the water-soluble, cell-impermeable, biotin analogue sulfo-NHS-biotin for 30 min at 4°C in PBS-CM. The biotinylation reaction was quenched with 50 mM NH₄Cl in PBS-CM followed by two washes with cold PBS. The cells were then ready for Triton X-114 phase separation.

Triton X-114 Extraction and Immunoprecipitations. Biotinylated and nonbiotinylated TPA-treated and control MCF10A cells were solubilized with 1.5% Triton X-114 in TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 1 mM CaCl₂, 1 mM MgCl₂, 1 mM PMSF, 10 μ g/ml aprotinin, 1 μ g/ml leupeptin, 2 mM benzamide, and 5 mM EDTA. The extracts were centrifuged (15 min at 14,000 \times g at 4°C), and the supernatants were warmed (2 min at 37°C) and centrifuged (14,000 \times g at 22°C) to separate the lower, detergent and upper, aqueous phases. The aqueous phase was then incubated (1 h at 4°C) with gelatin-agarose beads followed by a brief centrifugation. The beads were then washed three times with cold TBS containing 5 mM CaCl₂ and 0.02% Brij-35, and the bound proteins were eluted in the same buffer containing 10% DMSO. After a brief centrifugation, the supernatant containing the eluted proteins was diluted (5-fold) with immunoprecipitation buffer (50 mM Tris buffer, pH 7.5, 150 mM NaCl, 0.05% Brij-35, and 1 mM PMSF). The samples were then incubated with various anti-MMP-9 antibodies or mouse or rabbit IgG, as controls, and protein G-Sepharose beads. The immunoprecipitates were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. After being blocked with blotto and washed with T-TBS, as described above, detection of the biotinylated proteins was accomplished using a HRP-conjugated streptavidin and the ECL detection system. The specificity of the detection was determined in nonbiotinylated cells extracted and immunoprecipitated as described above.

Subcellular Fractionation. MCF10A cells were grown in roller bottles (20 per isolation) to 80% confluence and then untreated or treated for 12 h with 100 nM TPA in serum-free medium. Control cells received vehicle alone. All of the following procedures were performed in the cold. The medium was aspirated and the cell monolayers were washed three times with cold PBS and scraped into PBS. After a 20-min centrifugation (1200 \times g) at 4°C, the pellet was resuspended in 150 ml of 25 mM Tris-HCl, pH 7.5, containing 8.5% sucrose, 50 mM NaCl, 5 mM EDTA, 10 mM NEM, 10 μ g/ml aprotinin, 1 μ g/ml pepstatin A, 1 μ g/ml leupeptin, and 1 mM PMSF. The suspension was homogenized in a Dounce homogenizer. The homogenate was centrifuged (10 min at 3000 \times g) and the supernatant (postnuclear fraction) was collected and centrifuged (140,000 \times g) in a SW28 rotor for 2 h at 4°C. The supernatant (cytosol) was collected, and the pellet (crude membranes) was resuspended in 10 ml of 25 mM Tris-HCl, pH 7.5, 50 mM NaCl buffer with protease inhibitors and applied to a discontinuous sucrose gradient (20/30/50/60%) in water. The samples were then centrifuged (140,000 \times g) in a SW28 rotor for 2 h at 4°C, and the 30–50% interface was collected and resuspended in the same buffers as described above and centrifuged again for 2 h at 140,000 \times g. The PM pellet was resuspended in the same buffer to a final concentration of 1.5–2.5 mg/ml. The enriched PM fraction was stored at –80°C or further purified in a Percoll gradient. To this end, 1 ml of PM (0.8 mg) was mixed with 9 ml of a solution of 33.3% Percoll in the same buffer. The mixture was then centrifuged (30,000 \times g) in a Ti 70.1 rotor for 30 min. The purified PM appeared as a visible band at a density of 1.048 g/ml and was collected and pelleted by centrifugation (100,000 \times g for 1 h). The enrichment of the preparation in PM

components was assessed by immunoblot analysis using antibodies to PMCA and MT1-MMP (47), two known PM proteins, and by measuring the activity of alkaline phosphodiesterase, a PM marker (50). Briefly, triplicate samples (20 μ l) of either homogenate, postnuclear fraction, cytosol, or PM were incubated (18 h at 37°C) with 250 μ l of substrate (10 mM thymidine 5'-monophosphate *p*-nitrophenyl ester). After incubation, the reaction was quenched with 1 ml of 0.4 M sodium carbonate in 0.22 M glycine, pH 10. The absorbance of the samples was determined in a spectrophotometer at a wavelength of 405 nm. Enzyme activity was expressed as absorbance unit/mg protein. These studies showed both PMCA and MT1-MMP were present in the enriched PM fraction and a 15-fold increase in phosphodiesterase activity compared to the postnuclear fraction.

To determine the nature of the association of MMP-9 with the PM, a PM aliquot (25 μ g of total protein) was resuspended in either PBS, 1 M NaCl, or 0.1 M Na₂CO₃, pH 11.5, and incubated for 30 min at 4°C. The samples were then centrifuged (1 h at 100,000 \times g), and the supernatant and pellet were collected followed by the addition of Laemmli sample buffer. For phase separation with Triton X-114, an aliquot (25 μ g of protein) of PM was incubated (15 min at 4°C) with Triton X-114 and centrifuged (15 min at 13,000 \times g and 4°C), and the pellet was washed twice with PBS. The supernatant was transferred to a new tube and then phase separated by a 2-min incubation at 37°C followed by brief centrifugation at room temperature to generate the aqueous and the detergent phase. Then, the aqueous, detergent, and pellet fractions were each mixed with Laemmli sample buffer followed by immunoblot analysis.

Pulse-Chase Analysis. MCF10A cells were grown to 80% confluence in 60-mm dishes and then treated (12 h) with 100 nM TPA in serum-free medium. The medium was aspirated and the cell monolayer was washed twice with warm (37°C) PBS followed by incubation (45 min) with 1 ml/dish of starving medium (DMEM without methionine supplemented with 25 mM HEPES). The cells were then pulsed with 500 μ Ci/ml of [³⁵S]methionine in starving medium (0.6 ml/dish) for 15 min at 37°C. After the pulse, the dishes were placed on ice, the medium was aspirated, and the cells were washed twice gently with PBS before addition of 1 ml/dish of chase medium (DMEM with 10% fetal bovine serum and 4.8 mM methionine). At the end of the chase period (0–120 min at 37°C), the medium was collected, centrifuged (5 min at 12,000 \times g), and transferred to a clean tube with the addition of harvest buffer (60 mM Tris-HCl, pH 7.5, containing 0.5% SDS, 2 mM EDTA, and 10 mM methionine, final concentrations). The samples were boiled (3 min), centrifuged, and transferred to a new tube with the addition of 5 mM iodoacetamide, 2.5% Triton X-100 and 20 μ g/ml aprotinin (final concentrations). The cell monolayers were washed twice with PBS and lysed in 0.5 ml/dish of warm harvest buffer. The lysates were then subjected to five cycles of boiling and freezing followed by a brief centrifugation. The supernatants were collected into new tubes with the addition of 5 mM iodoacetamide, 2.5% Triton X-100, and 20 μ g/ml aprotinin (final concentrations). For immunoprecipitations, the medium and lysate samples were incubated (16 h at 4°C) with 5 μ g of CA-209 anti-MMP-9 antibodies followed by the addition of 30 μ l of protein G-Sepharose beads for an additional 3-h incubation at 4°C. After the beads were recovered by a brief centrifugation, the supernatant was subjected to sequential rounds of immunoprecipitations with antibodies to TIMP-1, MT1-MMP, and TIMP-2. The recovered beads were washed (five times) with cold 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 0.1% NP-40, and 10% glycerol and resuspended in 15 μ l of Laemmli sample buffer with DTT followed by boiling (5 min). Samples of media and lysates were resolved by 8–16% SDS-PAGE. Detection of radiolabeled proteins was performed by autoradiography.

For coprecipitation of the MMP-9/TIMP-1 complex, an aliquot of the [³⁵S]-labeled medium from the 2-h chase period from the pulse-chase experiment was subjected to two consecutive rounds of immunoprecipitations with anti-MMP-9 and anti-TIMP-1 antibodies, respectively. To this end, the [³⁵S]-labeled medium, without boiling and without addition of harvest buffer, iodoacetamide, or Triton X-100, was incubated with anti-MMP-9 antibodies and protein G-Sepharose beads as described above. After the first centrifugation, the immunoprecipitates were processed as described above, and the supernatants were collected and subjected to another round of immunoprecipitation with anti-TIMP-1 antibodies using the same protocol. The immunoprecipitates were resolved by 8–16% SDS-PAGE under reducing conditions followed by autoradiography.

Endo-H Digestion. The immunoprecipitates with anti-MMP-9 from the biotinylated and pulse-chase samples were washed with TBS, centrifuged, and then resuspended in 25 μ l of 100 mM sodium phosphate buffer, pH 5.5, containing 50 mM 2-mercaptoethanol and 0.1% SDS. The samples were then boiled (5 min) and cooled, followed by the addition of 2 μ l of endo-H (25 units/ml). After incubation (2 h, 37°C), the samples were boiled in the presence of Laemmli sample buffer with reducing agents and subjected to SDS-PAGE followed by either streptavidin-HRP (biotinylated samples) or autoradiography (pulse-chase samples).

RESULTS

Surface Association of MMP-9 but not of TIMP-1 in TPA-treated MCF10A Cells. Cultured MCF10A cells secrete low amounts of latent MMP-9 and TIMP-1. Treatment of the cells with 100 nM TPA for a period as short as 2 h induced the expression and secretion of high amounts of latent MMP-9 and TIMP-1, as determined by gelatin zymography (Fig. 1A) and by immunoblot analysis of trichloroacetic acid-precipitated medium (Fig. 1B). Exposure of cells to TPA for more than 2 h did not alter the level of MMP-9 and TIMP-1 expression in the medium. Gelatin affinity chromatography of medium from TPA-treated cells followed by immunoblotting with antibodies to MMP-9 and TIMP-1 (Fig. 1C) demonstrated that the enzyme copurified with TIMP-1 consistent with complex formation. To further examine the medium for the presence of the complex, an immunoprecipitation of MMP-9 and TIMP-1 was carried out from [³⁵S]-labeled medium of TPA-treated MCF10A cells as described in "Materials and Methods." As shown in Fig. 1D, the anti-MMP-9 antibodies precipitated MMP-9 in complex with TIMP-1 (Lane 1). Subsequent immunoprecipitation of the same medium with anti-TIMP-1 antibodies showed presence of TIMP-1 (Lane 3), suggesting that free inhibitor remained after removal of the MMP-9/TIMP-1 complex and that MCF10A cells secrete excess inhibitor over enzyme after treatment with TPA. The small amount of MMP-9 detected with TIMP-1 (Fig. 1D, Lane 3) was not enzyme coprecipitated with the inhibitor because it could be detected in the control precipitate without

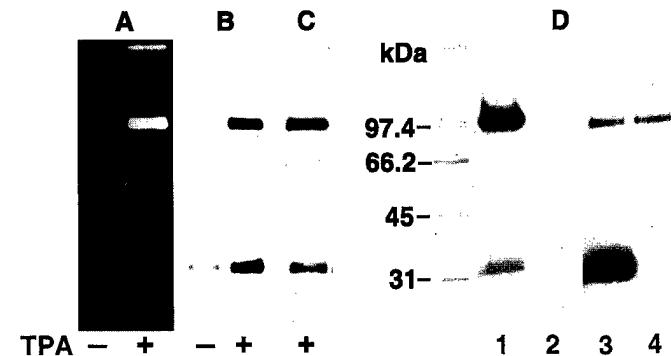


Fig. 1. Induction of MMP-9 and TIMP-1 expression in MCF10A cells by TPA. MCF10A cells were treated with 100 nM TPA (+) or vehicle alone (–) as described in "Materials and Methods." The medium was collected 18 h later and analyzed for MMP-9 expression by gelatin zymography (A) or immunoblot analysis (B and C). B, 200 μ l of medium from untreated and TPA-treated MCF10A cells was TCA precipitated, resolved in a 8–16% SDS-PAGE under reducing conditions, blotted to nitrocellulose, and analyzed for MMP-9 and TIMP-1 expression using both anti-MMP-9 and anti-TIMP-1 antibodies and the ECL detection system. C, immunoblot analysis of MMP-9 and TIMP-1 expression in medium from TPA-treated cells subjected to gelatin-agarose chromatography as described in "Materials and Methods." The eluted samples were subjected to 8–16% SDS-PAGE under reducing conditions and immunoblot analysis as in B. D, [³⁵S]-labeled medium of TPA-treated cells was immunoprecipitated with a polyclonal anti-MMP-9 antibody (Lane 1), followed by a second round of immunoprecipitation with a polyclonal anti-TIMP-1 antibody (Lane 3). Control samples received no antibodies (Lanes 2 and 4). Immune complexes were resolved by 8–16% SDS-PAGE under reducing conditions followed by detection by autoradiography. ¹⁴C-labeled molecular weight standards were used as reference.

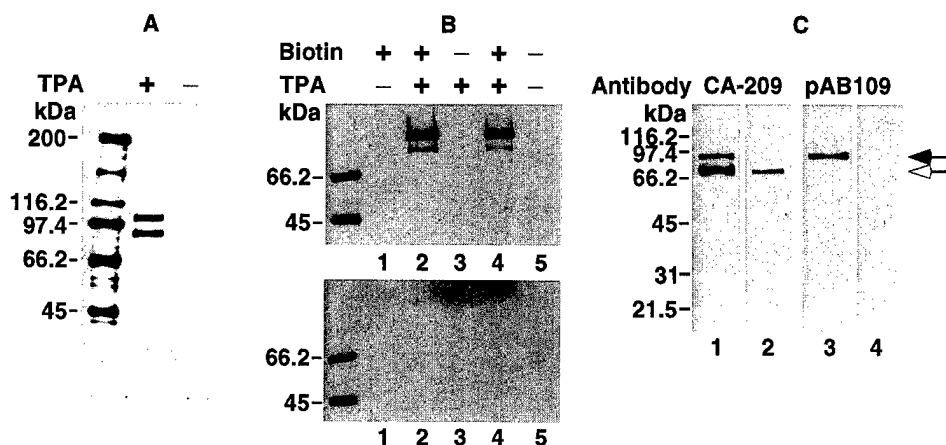


Fig. 2. Detection of MMP-9 in surface-biotinylated TPA-treated MCF10A cells. A, monolayers of MCF10A cells were treated (2 h) with 100 nM TPA (+) or vehicle alone (-) and then biotinylated with sulfo-NHS-biotin. After solubilization with Triton X-114, the aqueous phase was incubated with gelatin-agarose beads, and the eluted proteins were immunoprecipitated with anti-MMP-9 (CA-209) and protein G-Sepharose beads. The immunoprecipitates were resolved by 4–12% SDS-PAGE under reducing conditions, blotted to nitrocellulose paper, and developed with streptavidin-HRP and the ECL detection system. B, MCF10A cells treated with 100 nM TPA (+) or vehicle alone (-) were biotinylated (+) or not (-) and were processed as described in A. The samples were then immunoprecipitated with either CA-209 (Lanes 1–3 and 5, top panel) or pAB109 (Lane 4, top panel) or without antibodies (bottom panel) and subjected to 8–16% SDS-PAGE under reducing conditions, blotted to nitrocellulose paper, and developed with streptavidin-HRP and the ECL detection system. C, recombinant MMP-9 was either partially activated with 1 mM APMA (Lanes 1 and 3) to obtain a mixture of latent (M_r 92,000) and active (M_r 82,000) forms or activated to the M_r 82,000 form by stromelysin 1 (Lanes 2 and 4) as described in “Materials and Methods.” The enzymes were subjected to 8–16% SDS-PAGE under reducing conditions and blotted to nitrocellulose paper. The blots were incubated with either pAB109 (against the NH_2 -terminal domain) or monoclonal antibodies to MMP-9 (CA-209). Immune complexes were detected with the ECL detection system. C, filled arrow, latent MMP-9; open arrow, active form.

antibody (Fig. 1D, Lane 4) and represented MMP-9 remaining in the sample after the first immunoprecipitation.

To determine whether MMP-9 and TIMP-1 were associated with the cell surface, control and TPA-treated MCF10A cells were surface biotinylated and subjected to phase partitioning with Triton X-114. The resultant aqueous phase was incubated with gelatin-agarose beads, and the eluted proteins were immunoprecipitated with anti-MMP-9 antibodies. As shown in Fig. 2A, CA-209 antibodies precipitated a biotinylated M_r 92,000 protein from the extracts of TPA-treated MCF10A cells but not from the untreated cells consistent with cell surface localization of MMP-9. The CA-209 antibodies also precipitated a biotinylated protein with a M_r of approximately 85,000 from the TPA-treated cells (Fig. 2A). Although the M_r 85,000 form was consistently biotinylated, its intensity varied in different experiments. The amount and pattern of cell surface biotinylated MMP-9 were not affected by the duration of the TPA treatment (2–18 h). A biotinylated protein with the molecular weight of TIMP-1 ($\sim M_r$ 31,000) could not be detected under these conditions (Fig. 2A). The lack of TIMP-1 coprecipitation with MMP-9 from the surface-biotinylated cells was not due to dissociation of the complex during phase separation with Triton X-114 because TIMP-1 could be readily co-immunoprecipitated with MMP-9 when culture medium or a purified recombinant MMP-9/TIMP-1 complex was subjected to the same extraction procedure (data not shown). Direct immunoprecipitation of TIMP-1 from lysates of surface-biotinylated MCF10A cells was unreliable due to the presence of multiple nonspecific proteins coprecipitating with the anti-TIMP-1 antibodies (data not shown).

To determine whether the biotinylated M_r 85,000 form was activated MMP-9, we carried out an immunoprecipitation of surface-biotinylated cells with CA-209 antibodies and with a polyclonal antibody (pAB109) directed against the NH_2 -terminal prodomain of MMP-9 (Fig. 2B). The results of this experiment showed that the immunoprecipitation pattern obtained with the pAB109 antibodies (Fig. 2B, Lane 4, top panel) was identical to that obtained with CA-209 (Fig. 2B, Lane 2, top panel), suggesting that the M_r 85,000 species also contains the complete NH_2 -terminal prodomain. This finding and the fact that all of the procedures used to extract the cell surface associated MMP-9 were carried out in the presence of EDTA

suggest that the M_r 85,000 species is a latent MMP-9 isoform. Detection of the biotinylated M_r 92,000 and 85,000 MMP-9 forms was specific because immunoprecipitations of either untreated, nonbiotinylated (Fig. 2B, Lane 5, top panel) or TPA-treated, nonbiotinylated (Fig. 2B, Lane 3, top panel) MCF10A cells did not produce signals. In addition, no positive signals were detected with samples subjected to the same protocols but without addition of antibodies (Fig. 2B, bottom panel). The ability of the two MMP-9 antibodies to recognize either latent or active MMP-9 was confirmed by immunoblot analysis, and CA-209 recognized both active and latent forms (Fig. 2C, Lanes 1 and 2), whereas pAB109 reacted only with latent enzyme (Fig. 2C, Lanes 3 and 4), as expected.

MMP-9 Is Present in the PM of TPA-treated MCF10A Cells. Because MMP-9 but not TIMP-1 was detected on the surface of TPA-treated MCF10A cells, we wished to determine whether MMP-9 could also be identified on isolated PM. As shown in the zymogram in Fig. 3A, the PM fractions contained M_r 92,000 and 85,000 gelatinolytic forms that were identified as MMP-9 by immunoblot analysis

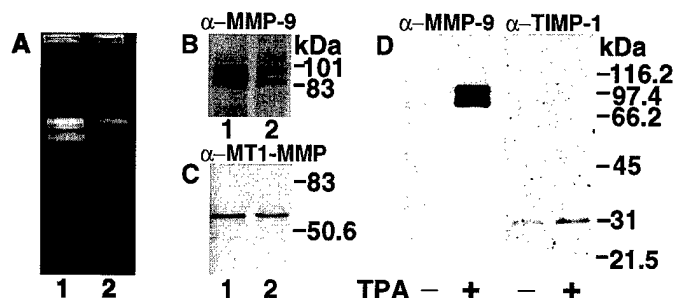


Fig. 3. Localization of MMP-9 in enriched-PM fractions of MCF10A cells. Gelatin-zymography (A) and immunoblot analysis (B–D) of PM isolated from MCF10A cells treated with TPA (A–C and + in D) or vehicle alone (D, -) by sucrose gradient (A–C, Lane 1 and D) and by Percoll gradient (A–C, Lane 2). PM fractions (10 μ g/lane) were subjected to gelatin-zymography as described in “Materials and Methods.” For immunoblot analysis, enriched-PM fractions (B and C, 10 μ g/lane; D, 45 μ g/lane) were subjected to 8–16% SDS-PAGE under reducing conditions and blotted to nitrocellulose paper for detection with either anti-MMP-9 (CA-209; B and D), anti-TIMP-1 (C), or anti-TIMP-1 (D) antibodies. D, note that the exposure time of the film with the anti-TIMP-1 antibodies was 15 min, whereas that of the anti-MMP-9 antibodies was 1 min.

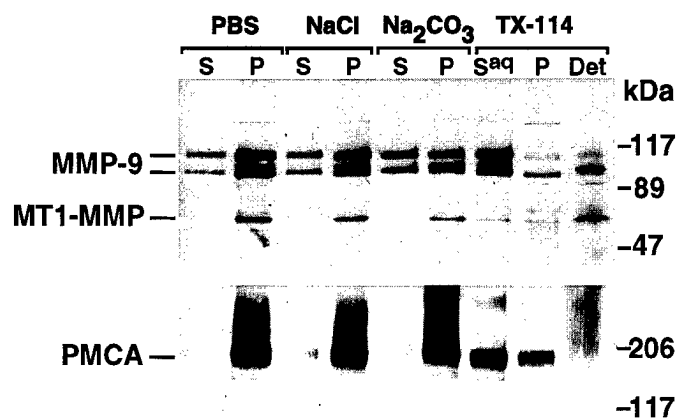


Fig. 4. Analysis of the PM association of MMP-9. Enriched PM fractions (25 μ g) from TPA-treated MCF10A cells were treated with either PBS, 1 M NaCl, or 0.1 M Na_2CO_3 or subjected to phase separation with Triton X-114. After PBS, 1 M NaCl, and 0.1 M Na_2CO_3 treatments, the PM suspensions were centrifuged to obtain the supernatant (S) and pellet (P). Triton X-114 separation of the PM into the aqueous (S^{aq}) and detergent (Det) phases and insoluble pellet (P) was achieved as described in "Materials and Methods." The samples were resolved by SDS-PAGE under reducing conditions followed by immunoblot analysis with anti-MMP-9, anti-MT1-MMP, and anti-PMCA antibodies.

(Fig. 3, B and D). Both CA-209 and pAB109 antibodies recognized the MMP-9 forms as shown with the surface-biotinylated enzymes (Fig. 2B), suggesting that both MMP-9 forms on the PM represent latent enzymes. In some occasions, the M_r 85,000 form appeared as a doublet; however, this was inconsistent. The zymogram also showed a high molecular weight ($\sim M_r$ 200,000) gelatinolytic band, possibly representing the MMP-9 homodimer. No differences were observed in PM preparations that were further purified by Percoll gradient. As a control, the PM fractions were subjected to immunoblot analysis for expression of MT1-MMP (Fig. 3C). Association of MMP-9 with the PM was only shown after TPA-treatment (Fig. 3D). In contrast, TIMP-1 was barely detected in the PM of untreated and TPA-treated cells (Fig. 3D), in agreement with the lack of detection of TIMP-1 in the surface of the biotinylated cells.

The nature of the association of MMP-9 to the PM was examined by subjecting the PM fraction to various treatments. After treatment, the PM suspension was centrifuged, and the pellet and the supernatant were examined for the presence of MMP-9 by immunoblot analysis. As a control, these fractions were also examined for the presence of PMCA and MT1-MMP. As shown in Fig. 4, extensive washes of the PM with PBS or treatment with 1 M NaCl released only a low amount of MMP-9 into the supernatant, whereas most of the enzyme remained bound to the pellet after equal proportions of each sample were analyzed. Treatment of the PM with 0.1 M Na_2CO_3 , pH 11.5, showed an apparently equal distribution of MMP-9 between the supernatant and the pellet. Neither PMCA nor MT1-MMP was released from the PM by these treatments, as expected. Phase partitioning of the PM preparation with Triton X-114 showed that most of the MMP-9 forms were released into the aqueous phase (Fig. 4, S^{aq}). However, a small amount, containing mostly the M_r 85,000 form, remained in the detergent phase (Det) and in the insoluble pellet (P). Fig. 4 also shows the effects of these treatments on the distribution of MT1-MMP and PMCA, which demonstrated that these two proteins behave like integral PM proteins with the exception of the reported anomalous distribution of PMCA into the aqueous phase after phase separation with Triton X-114, as expected. Taken together, these results suggest that PM-associated MMP-9 behaves like a peripheral membrane protein. However, the low molecular weight form of MMP-9 remained strongly bound to the PM and could not be extracted by any of these treatments.

Pulse-Chase Analysis of MMP-9 Synthesis in TPA-treated MCF10A Cells. Because two MMP-9 latent forms were biotinylated on the cell surface and bound to the PM, whereas only the M_r 92,000 form was found in the culture medium, we investigated the biosynthetic pathway of MMP-9 in TPA-treated MCF10A cells by pulse-chase analysis. The synthesis of MMP-9 was compared with that of TIMP-1, TIMP-2, and MT1-MMP (Fig. 5). After a 15-min pulse, the intracellular (cellular fraction) and extracellular (medium) fractions were harvested at various times (0–120 min) and immunoprecipitated with the appropriate antibody. As shown in Fig. 5, a protein of M_r 83,000–85,000, probably representing the precursor form of MMP-9, was detected at the 0-min chase in the cell lysate. After a 15-min chase, the M_r 85,000 MMP-9 precursor form was gradually converted to the mature enzyme (M_r 92,000), consistent with processing of complex oligosaccharide chains. Both forms were clearly detectable intracellularly even after a 120-min chase. Analysis of the medium (Fig. 5, extracellular panels) from the same experiment demonstrated a gradual secretion of the M_r 92,000 proenzyme starting after the 30-min chase, in agreement with the time (15 min) of processing of the precursor form into the mature proenzyme (M_r 92,000). In the medium, the presence of low molecular weight forms of the enzyme could not be detected.

The biosynthetic pathway of MMP-9 in TPA-treated cells was compared to that of TIMP-1, TIMP-2, and MT1-MMP. The TIMPs, like MMP-9, are secreted proteins, whereas MT1-MMP is membrane bound. As shown in Fig. 5, the TIMP-1 precursor form was readily detected at time 0 and was gradually glycosylated and secreted from the cells after a 60-min chase. After 90 min, TIMP-1 was barely detected intracellularly. It should be noted that the denaturing conditions of the pulse-chase protocol do not allow for determination of complex formation in the intracellular and extracellular compartment. TPA-treated MCF10A cells also produce TIMP-2, which was rapidly synthesized and secreted. However, in contrast to TIMP-1, a fraction of TIMP-2 remained in the intracellular compartment even after a 6-h chase (data not shown). Pulse-chase analysis of the biosynthetic pathway of MT1-MMP showed that 15 min after synthesis, the proenzyme form (M_r \sim 65,000–63,000) was converted to a lower molecular weight form (M_r \sim 60,000), consistent with intracellular processing. This form was not further processed and remained cell associated. Taken together, these studies demonstrated a unique processing of MMP-9 compared to that of TIMP-1, in which both MMP-9 forms remained associated with the cell compartment, whereas only the mature form was secreted.

Endo-H Digestion of Intracellular and Cell Surface MMP-9. Because MMP-9 is a glycosylated enzyme (25), we examined the glycosylation pattern of the cell surface-associated and intracellular forms of MMP-9 by endo-H digestion. This enzyme specifically cleaves oligosaccharides of the high-mannose and hybrid type and helps to distinguish complex carbohydrates, usually present in the mature form of secreted glycoproteins, from high-mannose oligosaccharides. We postulated that the low molecular weight form of MMP-9 on the cell surface and PM fraction may be a differentially glycosylated MMP-9 latent form. We wished to determine the similarities and differences in glycosylation between the cell surface M_r 85,000 form detected after biotinylation and the intracellular M_r 85,000 precursor form, detected in the pulse-chase experiment. To this end, the immunoprecipitates of the surface-biotinylated cells (cell surface) and of the pulse-chase experiment (intracellular) collected after 0- and 30-min chase periods were subjected to endo-H treatment. Fig. 6 shows that the intracellular M_r 85,000 precursor form was endo-H-sensitive (Fig. 6, Lanes 2 and 4), consistent with the presence of N-linked high-mannose oligosaccharides, whereas the intracellular M_r 92,000 form was resistant (Fig. 6, Lane 4), consistent with the

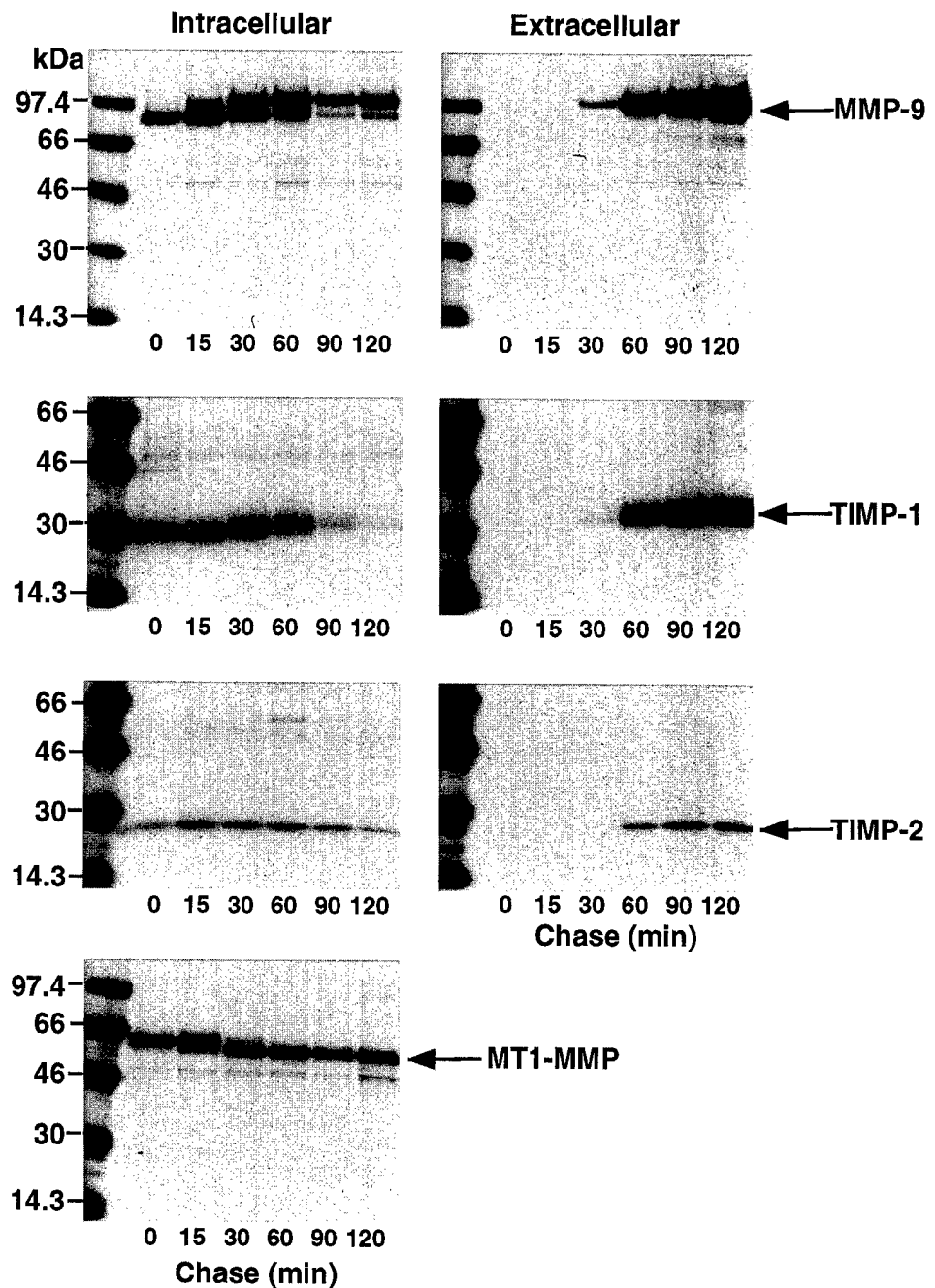


Fig. 5. Pulse-chase analysis of MMP-9, TIMP-1, TIMP-2, and MT1-MMP synthesis in TPA-treated MCF10A cells. Cells were treated with 100 nM TPA and subjected to pulse-chase analysis as described in "Materials and Methods." At the end of the chase period (0–120 min), the cells (intracellular) and the medium (extracellular) were subjected to consecutive rounds of immunoprecipitations with antibodies to MMP-9, TIMP-1, MT1-MMP, and TIMP-2. The immunoprecipitates were subjected to 8–16% SDS-PAGE analysis under reducing conditions followed by autoradiography. ^{14}C -labeled molecular weight standards were used as reference.

addition of complex carbohydrates to the mature M_r 92,000 form in the Golgi complex. Endo-H digestion of the surface-biotinylated MMP-9 forms revealed that the M_r 85,000 enzyme was endo-H sensitive, whereas the M_r 92,000 form was resistant (Fig. 6, Lane 6). Consistently, secreted MMP-9 was also endo-H resistant (data not shown). Taken together, these results show that the cell surface-associated M_r 85,000 MMP-9 latent form possesses a glycosylation pattern that is similar to that of the intracellular M_r 85,000 precursor form. Thus, the cell surface-associated M_r 85,000 form probably represents a unique processing and targeting to the cell surface of the precursor form of MMP-9 in TPA-treated MCF10A cells.

DISCUSSION

In this study, we present evidence on the cell surface association of MMP-9 in human MCF10A breast epithelial cells treated with TPA.

This was demonstrated by immunoprecipitation with anti-MMP-9 antibodies of surface-biotinylated MCF10A cells that revealed the presence of two MMP-9 forms with molecular weights of approximately 92,000 and 85,000 on the cell surface. Immunoprecipitation of the surface-biotinylated cells with an antibody directed against the NH_2 -terminal propeptide of MMP-9 demonstrated that both enzymes contain the complete propeptide domain and consequently are in the latent form. Thus, in TPA-treated MCF10A cells, appearance of the M_r 85,000 form on the cell surface is not the result of proenzyme activation. Consistent with these results, the two MMP-9 forms were also identified in an enriched PM fraction of TPA-treated cells. To elucidate the nature of the M_r 85,000 form on the cell surface, we carried out pulse-chase analysis experiments and endo-H digestion of precursor and mature forms of MMP-9. These studies showed a gradual intracellular processing of the precursor form (M_r 85,000) of

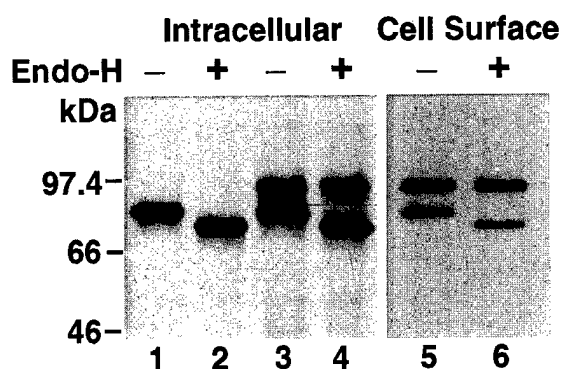


Fig. 6. Endo-H digestion of intracellular and cell surface-associated MMP-9. Immunoprecipitates of the pulse-chase samples (Lanes 1 and 2, 0-min chase; Lanes 3 and 4, 30-min chase) with anti-MMP-9 antibodies or of surface-biotinylated (Lanes 5 and 6) TPA-treated MCF10A cells were digested with (Lanes 2, 4, and 6) or without (Lanes 1, 3, and 5) endo-H as described in "Materials and Methods." The samples were resolved by 7.5% SDS-PAGE analysis under reducing conditions followed by detection by autoradiography (intracellular) or streptavidin-HRP and the ECL detection system (cell surface).

MMP-9 into the mature enzyme. Whereas the latter were secreted into the medium, the M_r 85,000 form remained in the cellular fraction. Incomplete glycosylation was unlikely to be the cause of impaired secretion of the M_r 85,000 form because in the presence of tunicamycin, a glycosylation inhibitor, MCF10A cells secreted a lower molecular weight form of latent MMP-9.⁴ This suggests that the M_r 85,000 species of MMP-9 was retained on the PM by a unique interaction. The pulse-chase studies also showed significant differences between the processing of MMP-9 and TIMP-1. Whereas the precursor form of TIMP-1 was rapidly processed and secreted into the medium, both the precursor MMP-9 form and a significant portion of the mature enzyme remained cell associated. Similar to MMP-9, TIMP-2 showed a codistribution between the cells and the medium, suggesting that the presence of TIMP-2 in the cellular fraction, after a 2-h chase period, was the result of secreted inhibitor that subsequently bound to the surface possibly via MT1-MMP (10, 47). Indeed, MCF10A cells produce MT1-MMP and after synthesis this enzyme was intracellularly processed to a lower molecular weight form that remained in the cellular compartment. Endo-H digestion of the intracellular and cell surface-bound MMP-9 enzymes demonstrated that both the intracellular and the cell surface M_r 85,000 forms were endo-H sensitive, whereas the mature M_r 92,000 enzyme was endo-H resistant. Thus, differences in molecular weight between the cell surface MMP-9 species are consistent with differences in glycosylation and are not likely the result of a COOH-terminal truncation of the surface M_r 85,000 form. Taken together, the results of the pulse-chase experiments and endo-H digestions were consistent with the M_r 85,000 form on the cell surface being the precursor form of MMP-9. The functional significance of the cell surface association of the M_r 85,000 precursor form remains unknown. However, a previous study showed that unglycosylated MMP-9 remains capable of autocatalytic processing and catalytic activity (52). Thus, the cell surface-bound M_r 85,000 form would be expected to be functionally competent and, as such, to participate in cell surface proteolysis. The localization of the M_r 85,000 form of MMP-9 on the cell surface may represent the targeting of the endo-H sensitive MMP-9 precursor form to the PM, a process likely to be related to alterations in intracellular processing and trafficking in response to TPA. In contrast, the cell surface association of the mature MMP-9 may represent enzyme binding after secretion.

In vitro expression of MMP-9 has been shown to be induced by

various cytokines (43) and growth factors (41, 42) and by cocultures of stroma cells with tumor cells (35, 44). Although most of the studies on MMP-9 induction examined the expression of the enzyme in the medium, a recent study with SKBR3 breast cancer cells cocultured with rat embryo fibroblasts showed that MMP-9 was not present in the detergent phase of Triton X-114 cell extracts (53). In agreement with these results, our studies showed that the M_r 92,000 MMP-9 form behaves like a peripheral membrane protein, because we were unable to detect it in the detergent phase. The hydrophilic nature of the MMP-9 association with the PM was also demonstrated after treatments of the PM with either 1 M NaCl, 0.1 M Na_2CO_3 , or Triton X-114. Yet, a readily detectable fraction of both MMP-9 forms could not be released from the PM. This was most evident with the M_r 85,000 species suggesting a tighter, likely hydrophobic interaction with the PM, although the molecular mechanism involved remains to be elucidated.

A unique property of latent MMP-9 is its ability to form a noncovalent complex with TIMP-1 (1, 12, 25, 36, 37, 40). This interaction is fundamental for the regulation of MMP-9 activation and catalytic activity. Analysis of the cellular distribution of TIMP-1 after TPA treatment of MCF10A cells indicated that little or no inhibitor was present on the cell surface. Indeed, TIMP-1 could not be detected in the immunoprecipitates of surface-biotinylated cells with anti-MMP-9 antibodies and was barely detected in the purified PM fractions, suggesting that neither of the surface-bound MMP-9 forms was forming a complex with TIMP-1. In contrast, immunoprecipitation with anti-MMP-9 antibodies and gelatin-agarose chromatography of the medium of the TPA-treated cells clearly demonstrated that TIMP-1 was in complex with MMP-9, as reported in previous studies (25, 40). Moreover, immunoprecipitation of the medium with the anti-TIMP-1 antibodies, after removal of the MMP-9/TIMP-1 complex, showed the presence of free TIMP-1, suggesting that the inhibitor was in molar excess of MMP-9. The low amounts of TIMP-1 detected on the PM were unlikely to be the result of inhibitor dissociation during sample preparation because mild conditions were used during subcellular fractionation. In fact, disruption of the noncovalent binding of TIMP-1 to MMP-9 requires harsh conditions and can be accomplished, among other methods, with 0.1% trifluoroacetic acid (54), 20 mM HCl,⁵ or 1% SDS. Also, a MMP-9/TIMP-1 recombinant complex could not be dissociated under the conditions used here to identify cell surface-associated MMP-9. The reason(s) for the lack of detection of a TIMP-1/MMP-9 complex on the cell surface in spite of the high level of TIMP-1 in the medium are presently unknown. It is possible that binding of MMP-9 to the cell surface may somehow interfere with TIMP-1 binding to the proenzyme form, suggesting that the association of MMP-9 with the cell surface may be mediated by the COOH-terminal domain. Alternatively, surface binding of MMP-9 may induce a conformational change, rendering its COOH-terminal domain inaccessible to the inhibitor. Further studies are required to distinguish between these possibilities. Nevertheless, the cell surface-association of MMP-9 free of TIMP-1 may have profound implications for activation and catalytic activity. Binding of TIMP-1 to the latent form of MMP-9 has been shown to alter activation by stromelysin-1 (37–39), plasmin (36, 37), and MMP-2 (45). Also, binding of TIMP-1 to activated MMP-9 inhibits enzymatic activity (37, 38, 45). Therefore, whereas in the extracellular space, the fate of latent MMP-9 would be determined by its interactions with TIMP-1, on the cell surface, the lack of available inhibitor would be expected to promote the activation and catalytic properties of MMP-9. It should be noted that in MCF10A cells, cell surface association of MMP-9 did

⁴ M. Toth, unpublished observation.

⁵ M. Olson, unpublished observation.

not result in enzyme activation, possibly due to the lack of activators in these cells. Thus, the binding of MMP-9 to MCF10A cells is not likely to be mediated by a PM-bound activator as reported with MMP-2 (10, 55). The cell surface association of MMP-9, however, may render the enzyme accessible to the action of MMP-9 activators present in the tissue or in neighboring cells (36, 37, 45, 50, 56, 57). Understanding the mechanisms that regulate the cell surface interactions of MMP-9 and elucidation of the molecule(s) responsible for binding would provide new insight on the regulation of ECM turnover in normal and malignant processes mediated by MMP-9.

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EPIDERMAL GROWTH FACTOR AND AMPHIREGULIN UP-REGULATE MATRIX METALLOPROTEINASE-9 (MMP-9) IN HUMAN BREAST CANCER CELLS

Sudhir B. KONDAPAKA, Rafael FRIDMAN and Kaladhar B. REDDY*

Department of Pathology, Wayne State University, Detroit, MI

The EGF family of proteins encompasses several polypeptides such as epidermal growth factor (EGF), transforming growth factor alpha (TGF α), amphiregulin (AR) and heregulin (HRG- β 1). These polypeptides regulate proliferation in breast cancer cells through interaction with membrane receptors. It has been previously shown that high EGF receptor number correlates with aggressive behavior and increased metastasis in human breast cancer. In the present study, we investigated the association between EGF and EGF-like ligand-induced DNA synthesis and secretion of MMP-9 and MMP-2 in metastatic SKBR-3 and non-metastatic MCF-7 breast cancer cells. Exposure of SKBR-3 cells to EGF or AR induces expression of MMP-9 but has no effect on MMP-2 secretion. In contrast to EGF and AR, HRG had no effect on gelatinase induction. None of the EGF polypeptides had any effect on gelatinase induction in MCF-7 non-metastatic breast cancer cells. While a relatively specific inhibitor of EGF receptor tyrosine kinase, PD 153035, inhibited EGF-, AR- and HRG-induced cell proliferation, it had no effect on MMP-9 induced by EGF and AR. Experimental evidence suggests that signaling mechanisms for cell proliferation and MMP-9 induction are mediated by different pathways down-stream of EGF receptor autophosphorylation or that low levels of EGF-induced signal that escape inhibition are sufficient to induce MMP-9 but unable to support cell proliferation. In addition, our results suggest that EGF and AR may modulate invasion of metastatic breast cancer cells by increasing the expression of MMPs. *Int. J. Cancer* 70:722–726, 1997.

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The EGFR and erbB2/HER2 receptor are members of the type I growth factor family of receptors whose over-expression has been shown to correlate with decreased disease-free survival and increased metastasis in advanced breast cancer (Sainsbury *et al.*, 1987; Khazaei *et al.*, 1993). Tumor cells generally exhibit a decreased requirement for growth factors when compared with their normal counterparts due to increased endogenous synthesis and secretion of polypeptide growth factors produced by the tumor itself and/or by the surrounding cells by an autocrine or a paracrine mechanism (Osborne and Arteaga, 1990). The over-expression of growth factors or their receptors would give growth advantage to the tumors expressing them, and indeed, this has been observed in many primary breast cancers and cell lines (Normanno *et al.*, 1994; Rajkumar and Gullick, 1994; Kokai *et al.*, 1989). These growth factors bind to specific plasma membrane receptors, activating the receptor tyrosine kinase activity that is essential for signal transduction and biological function (Reddy *et al.*, 1992; Heldin *et al.*, 1987; Kokai *et al.*, 1988). Furthermore, the over-expression of some of these mitogens can initiate *in vitro* transformation of both murine and human mammary epithelial cells (Ciardiello *et al.*, 1990, 1991).

Expression of MMPs, a family of zinc-dependent endopeptidases, has been associated with tumor cell invasion and metastasis due to the ability of these proteinases to hydrolyze a variety of extracellular matrix proteins. Two members of the MMP family, the 72-kDa (MMP-2, gelatinase A) and 92-kDa (MMP-9, gelatinase B) proteinases, have been shown to be highly expressed in breast tumors in both the stroma and the cancer cells (Monteagudo *et al.*, 1990; Davies *et al.*, 1993; Sato and Seiki, 1993). MMP-2 and MMP-9 are thought to play a role in the degradation of basement membrane collagen IV and, hence, may contribute to the invasive ability of breast cancer cells (Bae *et al.*, 1993). *In situ* hybridization studies have shown that the expression of MMP-2 mRNA is mostly localized in the tumor fibroblasts, while MMP-9 mRNA was found

to be expressed by epithelial cells and macrophages (Davies *et al.*, 1993; Polette *et al.*, 1993; Wilhelm *et al.*, 1989; Poulsom *et al.*, 1992; Fridman *et al.*, 1995; Okada *et al.*, 1995). Thus, several cellular constituents of the tumor may interact to carry out degradation of the extracellular matrix by modulating MMP expression. The mechanisms regulating gelatinase expression in breast tumors are unknown—in particular, those responsible for the expression of MMP-9 in breast cancer cells. Previous studies demonstrated that MMP-9 expression can be induced by a variety of stimuli, including phorbol ester (Wilhelm *et al.*, 1989), transforming growth factor- β , (Overall *et al.*, 1989) and interleukin-1 β (Conca *et al.*, 1989), in different types of tumor cells. Previously, it was also shown that EGF can increase the motility and invasiveness of keratinocytes, suggesting a role of EGF in partially supporting the tumor metastatic processes (Lisa and Laurie, 1996). The role of other EGF-like ligands in MMP-2 and MMP-9 expression in human breast cancer is not known. In the present study, we examined the effects of EGF-like ligands on MMP-9 and MMP-2 expression in metastatic SKBR-3 and non-metastatic MCF-7 breast cancer cells. Here, we show that exposure of breast cancer cells to EGF and AR, which bind to EGFR, resulted in EGFR autophosphorylation, increased cell proliferation and induction of MMP-9. In contrast to EGF and AR, HRG, which binds to erbB3 and erbB4 receptors, had no effect on MMP-9 expression. Also, PD 153035, a tyrosine kinase inhibitor which inhibits cell proliferation, failed to inhibit MMP-9 induction by EGF. Our results suggest that EGF and AR may contribute to the invasion of breast cancer cells by modulating expression of MMP-9.

MATERIAL AND METHODS

Cells and cell culture

The SKBR-3 and MCF-7 cell lines were obtained from the ATCC (Rockville, MD). All breast cancer cells were cultured in DMEM (GIBCO, Gaithersburg, MD), supplemented with 5–10% FCS (GIBCO) and 10 nM insulin (GIBCO), as described previously (Reddy *et al.*, 1994). All cell lines were routinely tested for Mycoplasma contamination and found to be negative.

Growth factors, antibodies and tyrosine kinase inhibitor

EGF and TGF α were purchased from Collaborative Research (Lexington, MA). AR was purchased from R&D Systems (Minneapolis, MN), and HRG was provided by Dr. Hung (M.D. Anderson, Houston, TX). The tyrosine kinase inhibitor PD 153035 was provided by Dr. Fry (Parke-Davis, Ann Arbor, MI). Tyrosine kinase inhibitor stock solutions were made in DMSO and diluted to appropriate concentrations in culture medium prior to addition to the cells. An equivalent dilution of DMSO (0.1%) without the inhibitor served as a control. Antibodies to EGFR and anti-

Abbreviations: TGF α , transforming growth factor alpha; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; AR, amphiregulin; HRG, heregulin; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase.

*Correspondence to: Department of Pathology, Wayne State University, 540 E. Canfield Avenue, Detroit, MI 48201, USA. Fax: (313) 577-0057.

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phosphotyrosine were purchased from UBI (Lake Placid, NY). MMP-9 and TIMP-1 polyclonal antibodies were provided by Dr. Fridman (Wayne State University, Detroit, MI).

Western blot analysis

SKBR-3 cells were grown to near-confluence in 15-cm dishes in regular growth medium. The seeding medium was removed, cells were washed twice with PBS and the medium was replaced with phenol red-free and serum-free DMEM. Twenty-four hours later, the cells were pre-exposed to 40 nM PD-153035 for 2 hr and then treated with EGF or AR for 5 min. Cells were then washed with ice-cold PBS and scraped into lysis buffer (50 mM Tris-HCl [pH 7.6], 100 mM NaCl, 2 mM EDTA, 1% NP-40, 1 mM orthovanadate, 0.5% sodium deoxycholate, 10 mg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin). After removal of cell debris by centrifugation (14,000 g, 30 min), the protein concentration was estimated. Equal amounts of protein from different groups were subjected to EGFR immunoprecipitation or immediately boiled for 5 min in sample buffer. Immunoprecipitates were washed 3 times with HNTG (20 mM Tris-HCl [pH 7.6], 10% glycerol, 0.1% NP-40, 150 mM NaCl) and heated in loading buffer for 10 min (100°C). Proteins were resolved in 8% SDS-PAGE and transferred onto nylon membrane (MSI, Westboro, MA), then probed with the appropriate antibody (Reddy *et al.*, 1992). Blots were washed 3 times with TTBS (20 mM Tris-HCl [pH 7.6], 0.05% Tween 20, 100 mM NaCl) and incubated with a secondary antibody coupled to horseradish peroxidase. After 3 washes, the blot was developed using the ECL chemiluminescence system (Amersham, Arlington Heights, IL). The level of phosphorylated EGFR was determined by densitometric scanning of M_r 170,000 band.

DNA synthesis

Cells were plated in 24-well tissue culture dishes (Corning, Corning, NY) at a density of 1×10^5 cells per well in DMEM with 10% FCS. After 24 hr, the seeding media were removed, the cells washed twice with PBS and the cells incubated in 0.1% BSA and phenol red-free DMEM (1 ml/well). Twenty-four hours later, growth factors and/or tyrosine kinase inhibitor were added. After 18 hr, [3 H]thymidine (82.3 Ci/mmol; NEN, Boston, MA; 0.25 μ Ci in a volume of 25 μ l) was added to each well for a 1 hr pulse. Cells were harvested, and the rate of DNA synthesis was estimated in triplicate by measuring the acid-precipitable radioactivity as described previously (Reddy *et al.*, 1994). Radioactivity was quantified in a Beckman (San Ramon, CA) LS 6000 liquid scintillation counter with an efficiency of 64%.

Gelatin zymography

Fifteen micrograms of protein from different groups were separated in 10% SDS-polyacrylamide gels co-polymerized with 0.1% gelatin. After electrophoresis, gels were rinsed twice in 2.5% Triton X-100 and incubated at 37°C for 20 hr in 1.5 M NaCl, 10 mM CaCl₂ and 50 mM Tris-HCl buffer (pH 7.5). Gels were stained with 0.5% Coomassie blue R250 and destained in 50% methanol and 10% acetic acid in H₂O. Gelatinolytic enzymes were detected as transparent bands on the blue background of Coomassie blue-stained gel.

RESULTS

EGF and AR induce MMP-9 in SKBR-3 breast cancer cells

We examined the effect of EGF, AR and HRG on the expression of MMP-2 or MMP-9 in SKBR-3 and MCF-7 cells. As shown in the zymogram of Figure 1a, untreated SKBR-3 cells do not secrete detectable levels of either MMP-2 or MMP-9. Exposure of these cells to as little as 5 ng/ml of EGF, however, resulted in secretion of the latent form of MMP-9 into the medium. Increasing EGF concentrations (50 and 500 ng/ml) did not change the level of MMP-9 secretion (Fig. 1a). Treatment of SKBR-3 cells with 1 ng/ml of AR also induced secretion of MMP-9 (data not shown). In contrast, HRG had no effect on MMP induction even after 72 hr (Fig. 1b). Neither of these mitogens induced MMP-2 expression in SKBR-3 cells or gelatinase expression in MCF-7 cells.

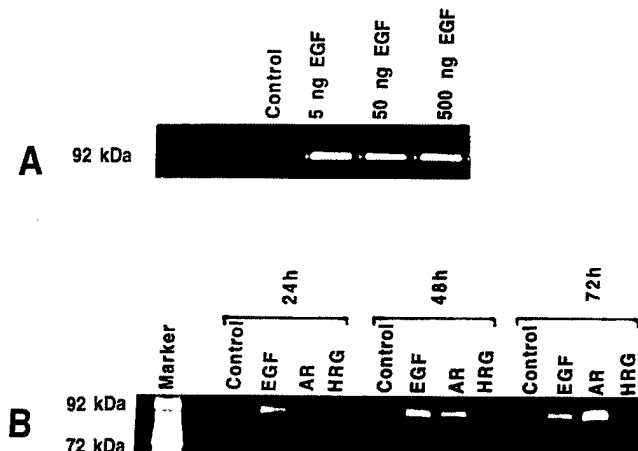


FIGURE 1 – EGF and AR up-regulate progelatinase B (MMP-9) in SKBR-3 breast cancer cells. SKBR-3 cells were grown in DMEM, with serum, to near confluence. The seeding medium was removed, cells were washed twice with PBS and medium was replaced with phenol red-free and serum-free DMEM. Twenty-four hours later, 50 ng/ml of EGF, AR and HRG were added to the culture. After an additional 24 hr, conditioned medium was collected and the presence of MMPs was evaluated by gelatin zymogram, as described in "Material and Methods". (a) Low concentrations of EGF (5 ng/ml) induce MMP-9. (b) Time course effects of EGF, AR and HRG. HRG had no effect on MMP-9 induction up to 72 hr.

Effect of EGF, AR and HRG on autophosphorylation of EGFRs and cell proliferation

To assess the functionality of EGF, AR and HRG on SKBR-3 cells, we examined the ability of these ligands to increase EGFR and erbB2 receptor autophosphorylation and cell proliferation. As shown in Figure 2a, both EGF and AR increased EGFR autophosphorylation in SKBR-3 cells when compared with untreated cells. In contrast, HRG had no effect. EGF and AR also caused phosphorylation of the erbB2 receptor, suggesting an EGF:erbB2 receptor heterodimerization. HRG, which predominantly binds to erbB3 and erbB4, marginally increased c-erbB2 receptor phosphorylation (Fig. 2a). In contrast to the differential effect of these ligands on type I receptor phosphorylation, they all promoted the proliferation of SKBR-3 cells (Fig. 2b).

Inhibition of EGFR autophosphorylation by a specific tyrosine kinase inhibitor

Previous studies demonstrated that PD 153035 is a relatively specific inhibitor of EGFR tyrosine kinase activity in a variety of target cells *in vitro* (Reddy *et al.*, 1992). We examined the effect of PD 153035 on EGFR phosphorylation in SKBR-3 cells. To this end, SKBR-3 cells were pre-incubated for 2 hr with PD 153035 prior to EGF (50 ng/ml) stimulation for 5 min and lysed. Western blot analysis using anti-phosphotyrosine antibodies demonstrated a dose-dependent inhibition of EGF-stimulated autophosphorylation of an M_r 170,000 band, representing EGFR (Fig. 3); half-maximal inhibition was observed at a concentration of about 40 nM of PD 153035.

Effect of PD 153035 on EGF- and AR-induced 3 H-thymidine incorporation and MMP-9 secretion

Since PD 153035 inhibited EGF-stimulated autophosphorylation, we examined its effect on EGF- and AR-stimulated 3 H-thymidine incorporation and on the ability of EGF to induce MMP-9 secretion. SKBR-3 cells were treated with EGF or AR in the presence or absence of 40 nM PD 153035 for 18 hr. Then, both the incorporation of 3 H-thymidine into the cells and the presence of MMP-9 in the media were examined. As shown in Figure 4a, both EGF and AR induced a 2- to 3-fold increase in 3 H-thymidine incorporation, which was blocked by PD 153035. Similar data

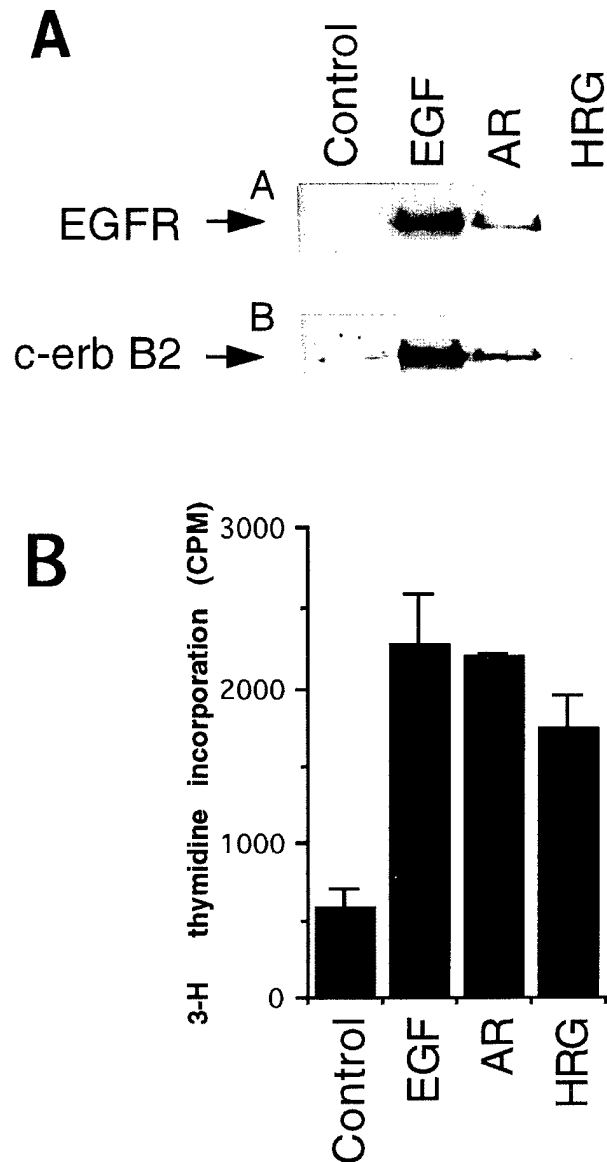


FIGURE 2 – Effect of EGF, AR and HRG on autophosphorylation of EGFRs and ^3H -thymidine incorporation. Cells were grown as shown in Figure 1; stimulated for 5 min with EGF, AR or HRG; and lysed. Western blot analyses were done by immunoprecipitating the protein lysate by anti-EGFR or anti-erbB receptor monoclonal antibodies, separated on SDS-PAGE and transferred to a nylon membrane, then probed with anti-phosphotyrosine antibody (a) (b) [^3H]thymidine incorporation by cells incubated for 18 hr with the ligands. Bars, mean \pm SE of triplicate determinations. EGF, AR and HRG show the expected pattern of receptor autophosphorylation and cell proliferation.

were obtained with MCF-7 cells (Fig. 5). Cells incubated in the presence of PD 153035 (40 nM) for 24 hr remain viable, as indicated by their morphological appearance, their lack of floating cells and their ability to exclude Trypan blue (>90% viable). Interestingly, secretion of MMP-9 into the media of SKBR-3 cells induced by treatment with either EGF or AR was not affected by the presence of PD 153035, as determined by both zymography and Western blot analysis with a specific MMP-9 antibody (Fig. 4b,c).

DISCUSSION

The majority of breast carcinomas when compared with normal breast epithelium show over-expression of ligands of the EGF

family, including TGF α , AR and cripto-1 (CR-1) (Normanno *et al.*, 1994). Furthermore, the introduction of rat or human TGF α cDNA into the germ line of transgenic mice promotes the appearance of hormone-dependent mammary adenocarcinomas (Matsui *et al.*, 1990; Jhappan *et al.*, 1990). These data support the hypothesis that growth factors are involved not only in cell proliferation but also in the pathogenesis of breast cancer. Accordingly, previous studies have shown that high levels of EGFR expression correlate with aggressive behavior and increased metastasis in human breast cancer (Sainsbury *et al.*, 1987; Khazaie *et al.*, 1993). Thus, expression of EGF-like ligands and their receptors in breast tumors may play a role in the expression of the metastatic phenotype. Our study was aimed at defining the role of EGF and EGF-like ligands and their receptors in the regulation of MMP-2 and MMP-9 in breast cancer cells differing in metastatic potential and in the number of EGFRs. SKBR-3 cells possess a high number of EGF, erbB2 and erbB3 receptors, while MCF7 cells possess a low number of EGFRs (Davidson *et al.*, 1987). Here, we have shown that as little as 5 ng/ml of EGF or 1 ng/ml of AR induce secretion of MMP-9 in SKBR-3 cells. The enzyme detected in the supernatant was in the latent form, as reflected by its molecular mass of 92 kDa. We detected up-regulation of the transcription factors AP-1[Fos/Jun] (data not shown), which are known to be stimulators of metalloproteinase genes (Sato and Seiki, 1993), but failed to detect mRNA levels of MMP-9 by Northern blot analysis. The effect of EGF and AR on MMP-9 secretion was specific for this enzyme since MMP-2 expression in SKBR-3 cells was not observed with these mitogens. This is consistent with the differential transcriptional regulation of MMP-9 and MMP-2 due to the presence of different promoter elements on the gelatinase genes (Brown *et al.*, 1990). The MMP-2 promoter has the characteristics of housekeeping genes and, as such, is constitutively expressed in many cultured cells. The promoter of MMP-9, like other members of the MMP family, contains, among other motifs, a TATA box and an AP1 element consistent with induction by a variety of cytokines, growth factors and oncogenes (Angel *et al.*, 1987).

Previous studies demonstrated stimulation of MMP-9 expression by EGF in keratinocytes (Lisa and Lurie, 1996) and colon carcinoma cells (Hyuga *et al.*, 1994). Thus, both normal and malignant cells produce MMP-9 in response to EGF. In our study, cell proliferation was significantly increased in response to EGF, AR and HRG in SKBR-3 and MCF-7 breast cancer cells. However, expression of MMP-9 in response to EGF and AR was induced in SKBR-3 but had no effect in MCF-7 cells. The reason for this difference is presently unknown. SKBR-3 and MCF-7 cells differ markedly in the number of EGFRs per cell. Thus, this difference may be related to the number of receptors. However, since EGF was able to induce MMP-9 in both normal and malignant keratinocytes (Lisa and Lurie, 1996), it is possible that the genetic background of each target cell may play a role in the differential effects of EGF and AR observed here. Nevertheless, these studies suggest that ligands inducing cell proliferation may also regulate other cellular activities associated with an invasive phenotype, including inducing enzymes involved in degradation of the extracellular matrix.

The proliferation or differentiation of breast cancer cells by EGF-like ligands is known to be mediated by type I receptors, including EGFR, erbB2, erbB3 and erbB4 depending on the cell lines (Peles and Yarden, 1993). Intriguingly, these presumably different pathways for growth and differentiation often use very similar intracellular signaling components in lower organisms (Doyle and Bishop, 1993). In SKBR-3 cells, interaction of HRG with erbB3 or erbB4 results in the transactivation of erbB2, with the concomitant stimulation of cell proliferation and lack of MMP-9 induction observed here. HRG, in contrast to EGF and AR, does not increase the autophosphorylation of EGFR. This suggests that MMP-9 induction may be predominantly mediated by EGFR-induced signal transduction.

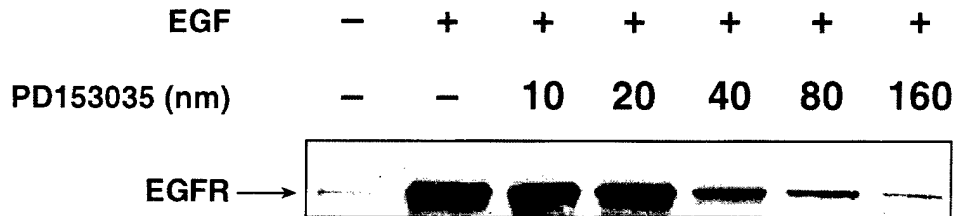


FIGURE 3 – Effect of PD 153035 concentration on autophosphorylation of EGFR in SKBR-3 cells. Cells were grown as shown in Figure 1, incubated for 2 hr with increasing concentrations of PD 153035, stimulated for 5 min with EGF (50 ng/ml) and lysed. EGFR phosphorylation was evaluated by Western blot analysis. Densitometric scanning of the autoradiogram suggests an LD₅₀ of around 40 nM of PD 153035.

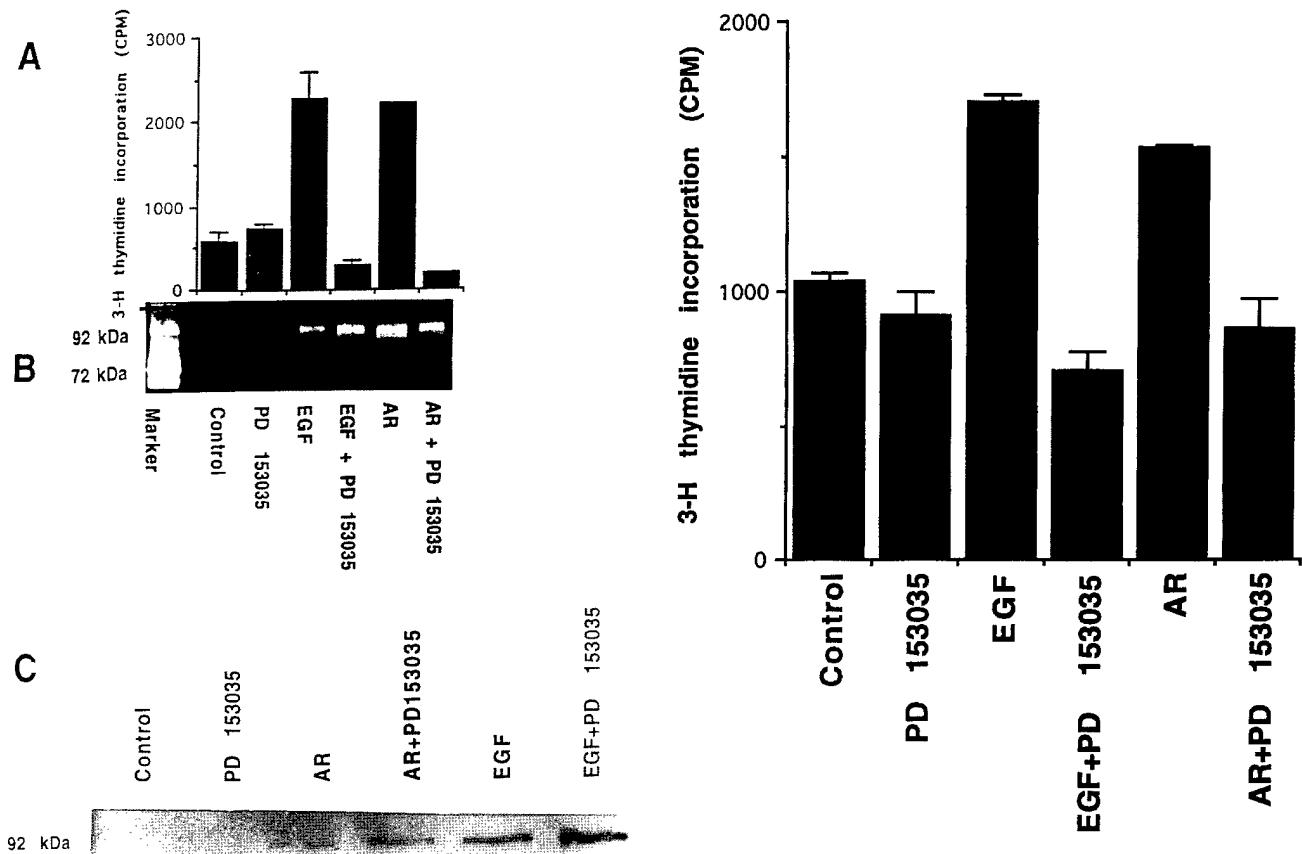


FIGURE 4 – Effect of PD 153035 on EGF- and AR-stimulated DNA synthesis and MMP-9 induction. SKBR-3 cells were grown in the presence of EGF and AR (50 ng/ml) with or without PD 153035. (a) [³H]thymidine incorporation in SKBR-3 cells after 18 hr of incubation. Bars: mean \pm SE of triplicate determinations. (b) MMP-9 induction by gelatin zymogram in the same condition as shown above. (c) Effects of PD 153035 on MMP-9 induction by Western blot analysis. PD 153035 significantly inhibited EGF- and AR-induced DNA synthesis but had no effect on the ability of EGF and AR to induce MMP-9.

To evaluate whether proliferation and MMP-9 were concurrently or independently acquired biological properties, we blocked cell proliferation with the specific EGF tyrosine kinase inhibitor PD 153035 (Fry *et al.*, 1994; Reddy *et al.*, 1992). PD 153035 was shown to be a relatively specific inhibitor of EGFR tyrosine kinase, and it suppresses autophosphorylation and selectively blocked EGF-mediated cellular processes including mitogenesis and early gene expression (Fry *et al.*, 1994). PD 153035 significantly inhibited EGF- and AR-induced cell proliferation (Fig. 4a) but had no effect on EGF- and AR-mediated induction of MMP-9 (Fig. 4b).

FIGURE 5 – Effect of PD 153035 on EGF- and AR-stimulated DNA synthesis. MCF-7 cells were grown in the presence of EGF and AR (50 ng/ml) with or without PD 153035. [³H]thymidine incorporation in MCF-7 cells after 18 hr of incubation. Bars: mean \pm SE of triplicate determinations. EGF and AR did not induce MMP-9 in MCF-7 cells.

The inability of PD 153035 to block EGF- and AR-induced MMP-9 in spite of its ability to inhibit cell proliferation suggests that the signaling mechanism for cell proliferation and MMP-9 induction may be mediated by different pathways down-stream of EGFR autophosphorylation. Another possibility is that low levels of EGF- or AR-mediated signals that escape inhibition may be sufficient for MMP-9 induction but are unable to support cell proliferation.

In conclusion, we have shown that exposure of SKBR-3 cells to EGF (5 ng/ml) or AR (1 ng/ml) induces expression of MMP-9. This suggests that malignant cancers with high levels of EGFRs may induce MMPs in response to EGF present in serum (20–27 μ M) (Hirata *et al.*, 1980) or tissue (1–5 ng/g tissue) (Hirata and Orth, 1979). Our results suggest that EGF and AR may modulate invasion of metastatic breast cancer cells by increasing the expression of MMPs.

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Phospholipase C- γ Immunostaining in Human Breast Carcinoma: Clinical Significance and Correlations with Protease and Growth-Factor Receptor Species

Daniel W. Visscher, M.D., Fazlul H. Sarkar, Ph.D., Timothy C. Kusinich, B.S.,
Rafael Fridman, Ph.D., Mousumi Banerjee, Ph.D., and Kaladhar B. Reddy, Ph.D.

*Department of Pathology, Harper Hospital, Karmanos Cancer Institute,
and Wayne State University School of Medicine, Detroit, Michigan*

■ **Abstract:** Cryostat sections of 73 invasive breast carcinomas were immunostained with a rabbit polyclonal antibody to phosphoinositide-specific phospholipase C- γ (PLC- γ), an enzyme that mediates signal transduction in tyrosine kinase growth-factor pathways. Degree of immunoreactivity was then correlated with clinicopathologic data (stage, ER status, recurrence) and immunostaining for tyrosine kinase growth-factor receptors (EGFR, ERBB-2) as well as selected "invasion-associated" proteases (cathepsin D, urokinase plasminogen activator, matrix metalloproteinases 2 and 9 [MMP-2, MMP-9]). Neoplastic epithelial populations were PLC- γ immunoreactive in 95% of tumors although staining was focally distributed (in <50% of cells) in 51% of positive cases. Forty-four percent also exhibited immunostaining of peritumoral, spindle-shaped cells (i.e., fibroblasts, endothelium, inflammatory cells). The degree of PLC- γ immunoreactivity in neoplastic epithelium was not significantly correlated with clinicopathologic features, growth factor receptor overexpression, or protease immunostaining. Stromal cell PLC- γ staining, however,

was significantly associated with stromal cell immunoreactivity for cathepsin D ($p = 0.03$), urokinase plasminogen activator ($p = 0.01$), and MMP-2 ($p = 0.04$). Disease recurrences were also more frequent in tumors with stroma/spindle cell PLC- γ immunoreactivity (66% vs. 41%, $p = 0.04$). We conclude that PLC- γ immunostaining, compatible with increased tyrosine kinase pathway signaling activity, is observed not only in a high proportion of neoplastic breast epithelial populations but also in accompanying stromal cell populations in a significant number of cases. Concordance with protease immunoreactivity among peritumoral stromal cells suggests that tyrosine kinase signaling may participate in protease elaboration in vivo, possibly conferring aggressive clinical behavior. ■

Key Words: breast carcinoma, phospholipase C- γ , proteases

Address correspondence and reprint requests to: Daniel W. Visscher, M.D., Department of Pathology, Harper Hospital, 3990 John R, Detroit, MI 48201, U.S.A.

Phosphoinositide-specific phospholipase C isoenzymes act as signal transducers for pathways that regulate cellular metabolism and differentiation. They hydrolyze phosphatidyl 4,5-biphosphate, generating diacylglycerol and inositol 1, 4, 5-triphosphate, which then act as second messenger species. The gamma-1 PLC isoenzyme

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(PLC- γ) is activated via phosphorylation by various tyrosine kinase growth factor receptors that are upregulated in malignant neoplasms, including epidermal growth factor receptor (EGFR), ERBB-2 and platelet-derived growth factor receptor (9,12,18). Accordingly, it has been shown that PLC- γ levels are increased in breast carcinomas, and that the activated (i.e., phosphorylated) form correlates with cellular levels of tyrosine kinase growth-factor receptors (1). Activity of PLC- γ is thus hypothesized to have a role in mediating or sustaining derangements of proliferation and differentiation that characterize malignant cells (1).

Our laboratory has recently shown that exposure of SKBR-3 metastatic breast-cancer cells *in vitro* to certain ligands of tyrosine kinase receptors (i.e., epidermal growth factor [EGF] and amphiregulin [AR]) induces expression of MMP-9 (gelatinase B) (10). Metalloproteinase induction, under these conditions, may be suppressed by inhibition of tyrosine kinase activity by inhibiting the binding of EGF or AR to its receptor by anti-EGFR antibodies (8,10), thus implying that PLC- γ may be one of the intermediate for cellular signaling involved in protease elaboration. The biological relevance of these findings is that proteolytic enzyme activity has been implicated as one of the factors that mediates steps of the metastasis cascade, such as invasive growth, stromal remodeling, and extravasation (2,17).

This study had two objectives. The first was to examine the distribution and clinicopathologic significance of PLC- γ immunostaining in a series of clinically derived human breast carcinomas. The second objective was to compare PLC- γ immunoreactivity with that of cellular proteases, including MMP-2 and MMP-9, cathepsin D (CD), and urokinase plasminogen activator (uPA) as well as tyrosine kinase receptors (EGFR and ERBB-2), which are often upregulated *in vivo* in human breast carcinomas. It was our hypothesis that PLC- γ overexpression may identify a subset of highly invasive breast neoplasms that are characterized by abundant protease elaboration.

MATERIALS AND METHODS

Immunostaining

We employed a set of 73 invasive breast carcinomas that had previously been analyzed immunohistochemically for epitopes corresponding to MMP-2 and MMP-9, uPA, CD, EGFR, and ERBB-2 (19,20). Cryostat sections for PLC- γ immunostaining (6 micron, from snap frozen slices, stored at -70°C) were mounted on gelatin-coated

slides and fixed in acetone. Immunostaining employed the avidin-biotin method (ABC Elite, Vector Laboratory, Burlingame, California) using a polyclonal antibody obtained from Santa Cruz Biotechnology, Santa Cruz, CA, as previously described by us, the specificity of which was established by Western blot analysis (as described below). Briefly, immunoprecipitated aliquots of cell fractions with anti-PLC- γ was subjected to SDS-PAGE and transferred to nitrocellulose membrane. Immunodetection of the isolated PLC- γ was done with anti-phosphotyrosine as previously described by us (15). For immunohistochemistry, incubation of the primary antibody was for 20 minutes at room temperature and incubation of the secondary antibody was for 10 minutes at room temperature. A buffer substitution slide was employed as a negative control. The distribution of immunostaining for all of the antibodies was assessed separately in neoplastic epithelial and in peritumoral, stroma-derived cells. An arbitrary semiquantitative scale was employed to grade immunostaining in both compartments: 0+ = <10% of cells positive, 1+ = 11–50% of cells positive, and 2+ = >50% of cells positive.

Clinical Data

Clinical stage and outcome data (chest wall or systemic disease recurrence, 50-month median follow-up, range 18–80 mo) were obtained from the SEER database at the Karmanos Cancer Institute, Detroit, Michigan, and estrogen receptor values (dextran charcoal method) were recovered from Harper Hospital Pathology Department archives. Twenty-eight patients (38%) were axillary node negative at presentation, 35 (48%) were node positive and 10 (14%) presented with presence of distant metastases. Forty-nine percent of the tumors were ER positive (defined as >15 f moles/mg). Comparisons between various clinical and immunostaining data were statistically evaluated using univariate analysis of contingency tables (chi-squared tests of association).

Characterization of Anti-PLC- γ Antibody

In order to confirm specificity of the PLC- γ antibody, we compared Western blotting of tissue lysates (from tissues that were shown to have differential immunopositivity) with lysates from A431 cells controlled for the expression of PLC- γ . The results are shown in Figure 1, which clearly show the presence of PLC- γ in A431 cells treated with EGF, whereas no PLC- γ was detected in untreated control. Tissue lysates also show PLC- γ positivity and the level of expression by Western blotting

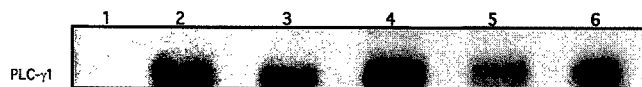


Figure 1. Immunoblotting of PLC- γ from human mammary tissues. Equal amounts of protein lysates were prepared and then subjected to immunoprecipitation with PLC- γ , antibodies linked to protein-A-sepharose. After separation of the immunoprecipitates by SDS-PAGE, the proteins were transferred to nylon membrane and immunoblotted with antiphosphotyrosine antibodies. The blots were then developed with ECL Western Kit from Amersham (Arlington Heights, IL). (Lane 1-protein lysate from A431, lane 2-lysate from A431 in the presence of EGF, and lanes 3 to 6 different tumors.)

was correlated with immunohistochemistry results. Immunohistochemical staining and Western blot analysis were done on tumors with similar stage and grading as judged by pathological examination. Four tumors were randomly selected based on the levels of PLC- γ immunostaining for Western blot analysis. Our results of immunostaining correlated with the protein levels in Western blot analysis.

RESULTS

Overall Immunostaining Results

Neoplastic epithelial populations were immunoreactive for PLC- γ in all but four cases (95%) although staining was focally distributed (i.e., 1+) in 51%. Immunostaining exhibited a granular cytoplasmic pattern with areas of cell membrane accentuation. Staining of *in situ* neoplasm, where present, was similar in degree to staining of invading tumor cell populations (see Fig. 2). Residual benign epithelium, when present, demonstrated focal weak immunoreactivity that was never as strong as the neoplastic cells (see Fig. 2).

In contrast to the neoplastic epithelial compartment, PLC- γ immunostaining of peritumoral stroma cells was observed in slightly less than half of the tumors (32/73, 44%). Further, stromal cell immunoreactivity was uniformly patchy (i.e., 1+ in distribution) within a given tumor. Positive cells often had a spindled configuration, like fibroblasts, and were intimately associated with the neoplastic populations, at the immediate periphery of tumor cell nests (see Fig. 2). Stromal cell PLC- γ immunoreactivity in residual benign tissue was limited to an occasional fibrocyte; confluent staining was not observed. There was no correlation between the degree of tumor-cell staining and presence of stromal-cell immunoreactivity (data not shown).

The proteases exhibited divergent patterns of immunoreactivity. Epitopes for MMP-2 and MMP-9 were observed primarily within neoplastic epithelial populations (MMP-2—75% of tumors positive, MMP-9—76% of tumors positive) with a minority of cases exhibiting stromal cell immunoreactivity (MMP-2—18% positive, MMP-9—11% positive). Urokinase plasminogen activator, in contrast, was observed more often within peritumoral spindled cells (50% stromal cell positive vs. 25% neoplastic epithelial positive). Cathepsin D immunoreactivity was observed frequently both in neoplastic epithelium (70% of tumors positive) and among stromal cells (88% of tumors positive). Neoplastic cell immunoreactivity for EGFR and ERBB-2 was observed in 37% and 22% of tumors, respectively.

Correlations Between PLC- γ Immunostaining and Other Parameters

Stromal cell immunoreactivity for PLC- γ is correlated with clinical data and abnormal oncogene expression in Table 1. Although stromal cell PLC- γ immunostaining was not significantly correlated with presence of ER, stage, or staining for either ERBB-2 or EGFR, recurrence was more likely among the positive cases. Sixty-six percent of (stromal cell) PLC- γ positive tumors recurred vs. only 41% of PLC- γ negative cases ($p = 0.04$, OR = 2.7). Table 2 compares stromal cell PLC- γ staining to cellular protease immunoreactivity. Stromal cell PLC- γ immunoreactivity was correlated significantly with stromal cell staining for cathepsin D ($p = 0.03$), MMP-2 ($p = 0.04$) and urokinase plasminogen activator ($p = 0.01$). There was no correlation between stromal cell immunoreactivities of PLC- γ and MMP-9. Interestingly, neoplastic cell immunostaining for cathepsin D and MMP-2 was more frequent in tumors with negative stromal cell PLC- γ staining. These trends, however, did not achieve statistical significance.

In contrast to stromal cell staining, the degree of neoplastic epithelial cell immunoreactivity for PLC- γ failed to correlate in a statistically significant way with any of the clinicopathologic parameters or proteases (see Table 3). Interestingly, presence of ER was actually more frequent in cases with 2+ PLC- γ staining than in cases with 1+ staining (56% vs. 47%). Similarly, the incidence of EGFR positivity in 1+ PLC- γ cases was 43% vs. 29% in 2+ PLC- γ cases. Finally, although cases with 2+ epithelial PLC- γ staining were more frequently positive for MMP-9 (85% vs. 71%), this trend also failed to reach statistical significance ($p = 0.14$).

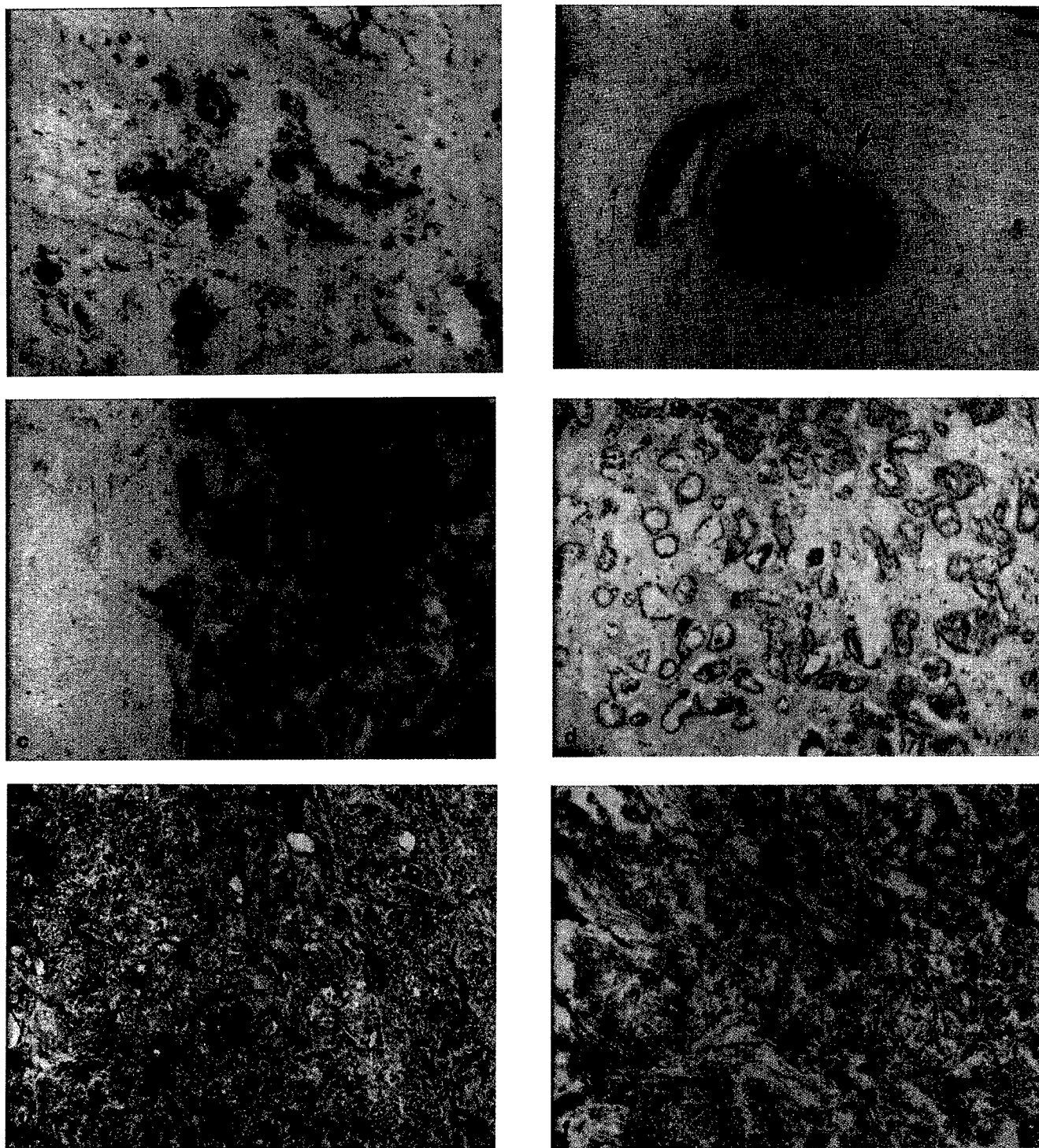


Figure 2. Immunostaining patterns of PLC- γ in breast tissue.

a- Residual benign breast tissue, demonstrating focal weak immunostaining ($\times 150$).

b- PLC- γ immunostaining in ductal carcinoma in situ (arrow). Note difference in immunostaining intensity from adjacent benign breast epithelial cells. Note also absence of immunostaining in surrounding stroma, which is relatively low in cellularity ($\times 150$).

c- Neoplastic cell immunoreactivity for PLC- γ . Note granular cytoplasmic immuno-reactivity with focal cell membrane accentuation ($\times 150$).

d- An invasive breast carcinoma with negative immunostaining ($\times 100$).

e- Stromal cell immunoreactivity for PLC- γ . Note the intimately peritumoral location of immunoreactive spindle cells ($\times 100$).

f- A higher magnification photomicrograph, demonstrating peritumoral immunoreactive spindle cells ($\times 200$).

Table 1. Association of Stromal Cell PLC- γ Immunostaining with Clinicopathologic Parameters in Breast Carcinoma

	Stromal cell PLC- γ		
	% Negative (0+) (n = 41)	% Positive (1-2+) (n = 32)	
ER			
negative	46%	58%	p = 0.32
positive	54%	42%	
STAGE			
node negative	39%	37.5%	p = 0.94
node positive	46%	50%	
distant	15%	12.5%	
ERBB-2			
negative	78%	78%	p = 0.99
positive	22%	22%	
EGFR			
negative	66%	59%	p = 0.57
positive	34%	41%	
OUTCOME			
disease-free	59%	34%	p = 0.04
recurrence	41%	66%	

OR = odds ratio.

DISCUSSION

The results of this study confirm previous observations demonstrating that neoplastic breast epithelium is characterized by overexpression of PLC- γ , as implied by increased immunoreactivity (1,16). Mechanistically, these findings likely reflect the elevated levels of signal transduction activity that characterize a malignant phenotype, in which proliferation and structural remodeling are both sustained as well as increased relative to normal. The degree of PLC- γ immunostaining in neoplastic epithelium, though, failed to correlate with abnormal expression of two tyrosine kinase growth-factor receptors (EGFR, ERBB-2) or with clinicopathologic markers of disease aggressiveness. It should be noted, in the former case, that there are a multiplicity of cellular tyrosine kinase signaling pathways in addition to those which are initiated by ERBB-2 or EGFR. Moreover, the observed trend toward greater PLC- γ staining among ER-positive and EGFR-negative tumors would imply that a shift toward estrogen-independent growth stimulation is not necessarily accompanied by increased expression of tyrosine kinase associated second messenger species.

Immunostaining for MMP-9 was more frequent among tumors with 2+, vs. 0-1+, PLC- γ immunoreactivity. However, due to lack of a statistically significant association, these data do not appear to confirm a predictable or direct relationship between evidence of more active tyrosine kinase signaling and upregulation of this

Table 2. Association of Stromal Cell PLC- γ Immunostaining with Protease Immunostaining in Breast Carcinoma

	Stromal cell PLC- γ		
	% Negative (0+) (n = 41)	% Positive (1-2+) (n = 31)	
CD Stromal			
negative	20%	3%	p = 0.03 OR = 7.8
positive	80%	97%	
CD Neoplastic			
negative	21%	41%	p = 0.06
positive	79%	59%	
MMP-2 Stromal			
negative	90%	71%	p = 0.04 OR = 3.7
positive	10%	29%	
MMP-2 Neoplastic			
negative	17.5%	35%	p = 0.08
positive	82.5%	65%	
MMP-9 Stromal			
negative	87.5%	90%	p = 0.77
positive	12.5%	10%	
MMP-9 Neoplastic			
negative	20%	29%	p = 0.38
positive	80%	71%	
uPA Stromal			
negative	63%	32%	p = 0.01 OR = 3.6
positive	37%	68%	
uPA Neoplastic			
negative	72%	79%	p = 0.7
positive	18%	21%	

OR = odds ratio.

particular protease species (i.e., as was observed in vitro). The data do not fully exclude the presence of such a relationship, though, since neoplastic cells in vivo are undoubtedly subject to a much more complex regulatory milieu than cell lines in vitro. Indeed, our data show that human breast carcinomas are extremely heterogeneous with respect to elaboration and distribution of proteases and growth factor receptors. Moreover, immunohistologic assessment of PLC- γ , as performed in this study, is semiquantitative at best and does not distinguish between the activated, versus inert, enzyme.

The best evidence from this study that implicates epidermal growth-factor family pathways in protease elaboration and tumor invasiveness are the immunostaining associations observed within the peritumoral spindle cell populations. Specifically, PLC- γ immunostaining of stromal cells was significantly correlated with staining for three proteases (cathepsin D, uPA, and MMP-2) among homologous populations. Our group and others have previously described peritumoral stromal cell distribution of protease epitopes (5,6,11,19,20). Recent studies have shown these peritumoral spindled cells to have features of myofibroblasts (13). Participation of such cells in tumor-associated protease activity is mech-

Table 3. Association of Neoplastic Epithelial PLC- γ Immunostaining with Various Parameters

	Neoplastic cell PLC- γ		
	% Negative (0+) (n = 4)	% Focally positive (1+) (n = 34)	% Diffusely positive (2+) (n = 34)
ER			
negative	100%	53%	44%
positive	0%	47%	56%
STAGE			
node negative	25%	34%	44%
node positive	75%	46%	47%
distant	0%	20%	9%
OUTCOME			
no recurrence	50%	43%	53%
recurrence	50%	57%	47%
ERBB-2			
negative	100%	80%	74%
positive	0%	20%	26%
EGFR			
negative	50%	57%	71%
positive	50%	43%	29%
CD (neoplastic)			
negative	25%	29%	30%
positive	75%	71%	70%
MMP-2 (neoplastic)			
negative	50%	26%	21%
positive	50%	74%	79%
MMP-9 (neoplastic)			
negative	50%	29%	15%
positive	50%	71%	85%

anistically logical since combined activity of multiple proteases, presumably derived from multiple sources, would theoretically be required for stromal remodeling given the molecular complexity of extracellular matrices. The potential role of epidermal growth-factor family signaling in protease regulation is further supported by the frequent presence of ligands, such as EGF and TGF- α as detected by immunostaining, among stromal cell populations (4,14).

The well-described biological and clinical relevance of angiogenic phenomena represent an analogous concept (3,7). We would not necessarily infer that protease elaboration is the only process mediated by tyrosine kinase pathway signaling. The so-called invasive phenotype is a composite of numerous functions including but not limited to migration, abnormal proliferation, and altered adhesion. In previous studies, we have demonstrated the adverse prognostic significance of stromal cell protease immunostaining (19,20). Thus, the observed association between stromal cell PLC- γ staining and disease recurrence could well represent a surrogate marker of inherently aggressive tumor behavior, as opposed to a causal association. It nevertheless underscores the biological

and clinical relevance of tumor-host interactions in neoplastic invasion and metastasis. Our results would suggest that breast carcinomas are not only characterized by a variable degree of stromal cell participation in tumor growth, but that those cases with "activated" stromal cell populations represent a clinically aggressive subset, possibly by virtue of protease activity.

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DENSITY-DEPENDENT REGULATION OF CELL-SURFACE ASSOCIATION OF MATRIX METALLOPROTEINASE-2 (MMP-2) IN BREAST-CARCINOMA CELLS

Suzanne MENASHI^{1,*}, Marie DEHEM², Isabelle SOULIAC¹, Yves LEGRAND¹ and Rafael FRIDMAN²

¹Unité 353 INSERM, Hôpital Saint-Louis, Paris, France

²Department of Pathology and Karmanos Cancer Institute, Wayne State University, School of Medicine, Detroit, MI, USA

Degradation of extracellular matrix takes place in areas of cell-matrix contacts and is partly carried out by the action of matrix metalloproteinases (MMP). MMP-2 is a member of the MMP family that has been associated with breast-cancer metastasis. In the present study, we investigated the association of MMP-2 to the surface of breast-cancer cells and revealed an MMP-2-binding site that is expressed on sparsely plated cells and which is progressively lost as the cells approach confluence. Gelatin zymography, immunostaining and flow cytometry of MDA-MB-231 cells from sparse cultures demonstrated binding both of latent and of activated exogenous MMP-2, while little or no binding of MMP-2 was observed in confluent culture. Analysis of the expression of MT1-MMP, TIMP-2 and αv integrin, 3 proteins shown to play a role in cell-surface association of MMP-2, revealed enhanced levels of these proteins in confluent MDA-MB-231 cells. Thus, the reduced MMP-2 binding to confluent cells is not related to a deficiency in these MMP-2-binding proteins. Taken together, these studies suggest that MMP-2 binding to the surface of breast-cancer cells is regulated by cell-cell interactions and that tumor cells invading from the main tumor mass can up-regulate their MMP-2-binding capacity to acquire greater invasive capacity. Int. J. Cancer 75:259–265, 1998.

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The process of metastasis formation is a complex multistep process involving detachment of tumor cells from the primary tumor, invasion through basal membrane into the surrounding stroma, intravasation into the circulatory system, extravasation at a distant site and outgrowth of a secondary tumor (Fidler, 1990). Many studies have shown that metastasis formation is partly associated with the expression and activity of a specialized group of extracellular-matrix (ECM)-degrading proteinases, the matrix metalloproteinases (MMPs). Among the members of the MMP family shown to play a role in tumor-cell invasion and metastasis are MMP-2 (gelatinase A/72-kDa type-IV collagenase) and MMP-9 (gelatinase B/92-kDa type-IV collagenase) (Tryggvason *et al.*, 1987; Stetler-Stevenson, 1990; Tryggvason, 1993). In various tumor systems, increased expression of MMP-2 has been associated with metastatic potential (Stetler-Stevenson, 1990). In human breast cancer, elevated MMP-2 protein (Tryggvason, 1993; Hoyhtya *et al.*, 1994) and mRNA (Polette *et al.*, 1993; Poulson *et al.*, 1993) levels have been detected compared to benign breast tissue. *In situ* hybridization studies have shown that MMP-2 mRNA in breast-cancer tissue is predominantly localized to the stroma (Polette *et al.*, 1993; Poulson *et al.*, 1993), while the enzyme was found to be localized on the surface of the invasive breast-cancer cells (Hoyhtya *et al.*, 1994; Visscher *et al.*, 1994). This suggests that MMP-2 is secreted by stroma fibroblasts and then binds to the surface tumor cells through a paracrine mechanism. Indeed, high-affinity MMP-2-binding sites have been demonstrated in MCF-7 and MDA-MB-231 breast-cancer cell lines (Emonard *et al.*, 1992). A correlation between a potential for surface binding and activation of MMP-2 and the metastatic capacity of breast-cancer cells have been observed both *in vitro* and in nude mice (Azzam and Thompson, 1992; Azzam *et al.*, 1993). To date, little is known regarding the biochemical interactions between MMPs and the cell surface. However, a novel membrane-type matrix metalloproteinase (MT1-MMP), localized on the cell surface via a transmembrane domain, has been described as the physiological activator of MMP-2 and has been implicated in the localization of MMP-2 on the cell

surface (Sato *et al.*, 1994, 1996; Strongin *et al.*, 1995). Interestingly, *in situ* hybridization studies of breast-cancer tissues demonstrated that the expression of MT1-MMP mRNA is confined to the tumor stroma (Okada *et al.*, 1995), raising the question as to whether MT1-MMP mediates the cell-surface association of MMP-2 in the breast-cancer cells. Studies with melanoma cells have suggested that the integrin $\alpha v \beta 3$ binds MMP-2 and thus may serve as a cell-surface receptor for this enzyme (Brooks *et al.*, 1996). It has also been suggested that the interaction between $\alpha v \beta 3$ and MMP-2 may promote cell motility and matrix degradation and thus contribute to the invasive behavior of the tumor cells (Brooks *et al.*, 1996).

The invasive phenotype of a tumor cell would also depend on the micro-environment of the tumor and metastatic tumor cells distant from the primary tumor are expected to have an enhanced invasive ability when compared with the cells of the main tumor mass. Differences in metastatic potential may be related, among other factors, to cell-cell interactions. It has already been shown, with C6 astrocytoma cells, that cells plated at low density released to the culture medium greater amounts of collagen-type-IV-degrading activity than in dense cultures (Tamaki *et al.*, 1996). Xie *et al.* (1994) have also shown that A431 human epidermoid-carcinoma cells secreted MMP-9 in response to cytokines and growth factors only when grown at low density. In the present study, we examined whether the cell-surface association of MMP-2 is influenced by cell density. We show that MDA-MB-231 breast-carcinoma cells exhibit enhanced MMP-2 binding when the cells are at low density and that this process is independent of the level of expression of MT1-MMP, TIMP-2 and αv integrin.

MATERIAL AND METHODS

Cells, enzymes, antibodies and chemicals

The metastatic breast carcinoma cell lines: MDA-MB231, MDA-MB435, ZR75-1, T47D and MCF7 were obtained from the ATCC (Rockville, MD) and cultured in DMEM supplemented with 10% FCS in a 95% air, 5% CO₂ atmosphere at 37°C. DMEM, penicillin, streptomycin, HEPES, PBS and heat-inactivated FCS were obtained from GIBCO (Cergy-Pontoise, France). BSA, fraction V, and Quick-Spin columns were obtained from Boehringer Mannheim (Meylan, France). Gelatin, Triton X-114, *p*-aminophenylmercuric acetate (APMA), protease inhibitors, and a monoclonal antibody (MAb) to β -actin (A-5441) were all purchased from Sigma (St. Louis, MO). Rhodamine-conjugated rabbit anti-mouse IgG, fluorescein-conjugated, anti-mouse, and non-immune mouse IgG were from Dako (Trappes, France). Human recombinant MMP-2 and TIMP-2 were expressed in a vaccinia-expression system in mammalian cells and purified to homogeneity as described (Fridman *et al.*, 1992). The MAbs to human MMP-2 (CA-801) and to human TIMP-2 (T2-101) have been described (Hoyhtya *et al.*, 1994). The rabbit polyclonal antibody to MT1-MMP was raised against a synthetic peptide (RFNEELRAVDSEY-PNIK) derived from the amino-acid sequence of human MT1-

*Correspondence to: Unité 353 INSERM, Hôpital Saint-Louis, 1 Avenue Claude Vellefaux, 75010 Paris, France. Fax: 33 1 5372 4027.
E-mail: s.menashi@chu-stlouis.fr

MMP (Gervasi *et al.*, 1996) and produced by Genetics Research (Huntsville, AL). MAb1960 to α_v integrin was purchased from Chemicon (Temecula, CA). RNeasy total RNA kit was purchased from Qiagen (Chatsworth, CA), Hybond-N from (Amersham, Aylesbury, UK), and Random Primers DNA labeling system (GIBCO BRL, Grand Island, NY). The enhanced-chemiluminescence (ECL) detection system, horseradish-peroxidase (HRP)-conjugated secondary antibodies and the BCA protein assay kit were purchased from Pierce (Rockford, IL).

Cell treatment and Triton X-114 extraction

To obtain MDA-MB-231 cells at different densities, 2 protocols were used. 1. Cells suspended in complete DMEM were seeded into 35-mm dishes at 5 different densities (5×10^4 , 1×10^5 , 2×10^5 , 4×10^5 , 1×10^6 cells/dish) and allowed to attach for 24 hr at 37°C before incubation with MMP-2; 2. Cells were seeded at a density of 5×10^4 cells/35-mm dish and allowed to grow in complete media. Cells with increasing density were obtained by collecting cells each day up to 4 days, when cells reached confluence. In both cases, the cells were washed twice with serum-free DMEM and then incubated (4 hr, 37°C) with 1 ml of the same serum-free medium containing 4 nM of recombinant latent MMP-2. After incubation, the cells were washed 3 times with PBS to remove unbound enzyme and lysed with 200 μ l of ice-cold 2% Triton X-114 in Tris-buffered saline (TBS: 50 mM Tris-HCl, pH 7.4, 150 mM NaCl) with the help of a rubber policeman. Amphiphilic cellular protein fraction containing integral membrane proteins was separated from hydrophilic proteins according to Bordier (1981). After centrifugation of the lysate at 10,000 g for 10 min to remove the Triton insoluble material, the supernatant was partitioned into detergent (lower) and aqueous (upper) phases by incubation at 37°C for 1 min followed by centrifugation at 1000 g for 5 min. The detergent phase was washed with 0.2 ml of cold TBS and repartitioned twice. After protein determination of the aqueous phase, fractions containing identical protein amounts were assayed by zymography (usually 5–10 μ g per well). In some experiments, sparse cultures of MDA-MB-231 cells were incubated with activated MMP-2. To this end, latent MMP-2 diluted in 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM CaCl_2 , 0.02% Brij-35 (collagenase buffer) was incubated (1 hr, 22°C) with 1 mM APMA (final concentration) prepared from a 10 \times APMA solution in 50 mM NaOH. To remove APMA, the active MMP-2 was applied to a Quick-Spin column previously equilibrated with collagenase buffer as described (Fridman *et al.*, 1995). The other breast-carcinoma cell lines, MDA-MB-435, ZR-75-1, T-47D and MCF-7, were seeded into 35-mm dishes at 2 different densities: 5×10^4 and 4×10^5 cells/dish, and allowed to attach in complete DMEM for 24 hr at 37°C, after which 60 to 80% confluence was obtained with the dense culture. After washing, the cells were incubated with 4 nM exogenous MMP-2 in serum-free medium, as described above for MDA-MB-231.

Gelatin zymography

Aliquots of cell lysates were assayed by substrate gel electrophoresis. Samples (10 μ g) were applied without heating or reduction to a 10% SDS-polyacrylamide gel containing 1 mg/ml of gelatin. After electrophoresis, the gel was washed twice for 20 min each time with 2.5% Triton X-100, followed by brief water washes, and incubated overnight at 37°C in 50 mM Tris buffer, pH 7.4, containing 5 mM CaCl_2 and 0.02% NaN_3 . The gel was stained with a solution of 0.1% Coomassie brilliant blue R-250 in 10% methanol and 5% acetic acid and then de-stained in 10% methanol and 5% acetic acid. Areas of protease activity were detected as clear bands against the blue-stained gelatin background.

Analysis of cell-associated MMP-2 by flow cytometry

Assays were performed on MDA-MB-231 cells in suspension harvested from monolayer cultures at day 1 and at confluence. Cells were grown for 18 hr (day 1) or 3 days (confluence) in DMEM containing 10% FCS. Cultures were then incubated in the presence of 4 nM recombinant MMP-2 in serum-free DMEM for 4

hr at 37°C and were washed 3 times with PBS to remove unbound enzyme. Cells were scraped into PBS with a rubber policeman, washed once more by centrifugation, and after resuspension in PBS they yielded a suspension of cells without any aggregates. Aliquots containing 1×10^6 cells/ml were incubated in PBS containing 10 μ g/ml of either non-immune mouse IgG or anti-MMP-2 (CA-801) antibodies for 1 hr at 22°C. The cells were then washed with PBS containing 0.2% BSA, incubated with fluorescein-conjugated anti-mouse IgG, washed again and finally re-suspended in PBS containing 1% paraformaldehyde. Staining was analyzed by a Becton Dickinson (Mountain View, CA) flow cytometer using FACScan research software.

Immunofluorescence staining

MDA-MB-231 cells were seeded on an 8-chamber tissue-culture dish (Lab-Teck, Nunc, Kampstrup, Denmark) and grown for 18 hr (day 1) or 3 days (confluence) in complete medium. Cells were then incubated with 4 nM of MMP-2 in serum-free medium for 4 hr at 37°C and were washed 3 times with PBS to remove unbound enzyme. Cells were fixed for 10 min with 4% paraformaldehyde in PBS, washed 3 times with PBS and incubated (1 hr, 22°C) with 15 μ g/ml of CA-801 diluted in PBS or non-immune mouse IgG. After being washed with PBS containing 0.2% BSA, cells were incubated with rhodamine-conjugated rabbit anti-mouse IgG for 45 min, washed again and mounted in a solution of 10% glycerol in PBS.

Northern-blot analysis

Total RNA was extracted from sparse and confluent cultures of MDA-MB-231 prepared according to protocol 2 described above, using the RNeasy Total RNA kit. RNA (20 μ g) from each sample was fractionated on a 1% agarose gel in the presence of formaldehyde, then transferred onto a nylon membrane, to which it was fixed using an optimized UV cross-linking procedure. The blot was pre-hybridized with a solution of 5 \times SSC, 50% formamide, 1% SDS, 5% Denhart's and 100 μ g/ml salmon sperm DNA (heated 5 min at 95°C) for 3 hr at 42°C, followed by hybridization at 42°C for 18 hr with a ^{32}P -labeled human cDNA probe for MT1-MMP (1.75 kb) or for TIMP-2 (1.1 kb) (a generous gift from Dr. G. Goldberg, Washington University, St. Louis, MO). The blot was then washed twice (20 min each time) with 0.1 \times SSC to 0.1% SDS at 55°C and autoradiographed at -80°C . RNA loading was normalized using the signal obtained with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe. Quantitation of signals was performed using an Ambis (San Diego, CA) Radioanalytic Imaging System.

Immunoblot analysis

Analysis of MT1-MMP protein expression was performed in sparse and confluent cells after phase partitioning with Triton X-114, as described above, to obtain the aqueous and the detergent phases. After measuring the protein concentration in the aqueous-phase samples by the BCA protein assay, samples of the detergent phase, calculated to contain equal protein content, were diluted 4-fold with distilled H_2O , after which Laemmli sample buffer was added. For TIMP-2 analysis, serum-free conditioned medium was collected after 16 hr from sparse and confluent cells, in accordance with protocol described above. The conditioned medium was concentrated by trichloroacetic-acid precipitation, followed by protein determination using the BCA protein assay. Equal protein amounts from each samples (40 μ g) were subjected to SDS-PAGE in reducing conditions, then transferred to a nitrocellulose membrane. The blot was blocked with 3% BSA and 3% non-fat dried milk in 100 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.02% NaN_3 for 16 hr at 4°C. The blot was washed twice for 15 min in TBS containing 0.1% Tween-20 (T-TBS) and incubated (1 hr, 22°C) with either anti-MT1-MMP antibody or anti-TIMP-2 antibodies diluted in T-TBS. After several washes with T-TBS, the blot was incubated with a HRP-conjugated goat anti-rabbit polyclonal antibody. Immunodetection of the antigen was performed using the ECL detection system according to the manufacture's instructions. Analysis of α_v -integrin expression was performed on sparse and

confluent cells, using protocols 1 and 2. Cells were lysed with 1 ml lysis buffer (25 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1% NP-40) with protease inhibitors (10 µg/ml aprotinin, 1 µg/ml leupeptin, 2 mM benzamidine and 1 mM phenylmethylsulfonylfluoride). The lysates were rocked at 4°C for 1 hr, then centrifuged (15 min, 13,000 × g). The supernatants were collected and, after measurement of protein concentration by the BCA assay, equal protein amounts (40 µg) were mixed with Laemmli sample buffer and reducing agents. Samples were subjected to SDS-PAGE and immunoblot analysis as described above, except that the primary antibody was anti-αv integrin. As a control for loading, the blot was also developed for detection of β-actin.

RESULTS

Cell-density-dependent binding of MMP-2 to MDA-MB-231 breast-carcinoma cells

To investigate the binding of MMP-2 to MDA-MB-231 cells as a function of cell density, the cells were plated at different densities and allowed to adhere in the presence of serum, after which they were washed and incubated with MMP-2 in serum-free medium. At the end of the incubation period, the cells were subjected to phase separation with Triton X-114 and the aqueous phase was examined for presence of MMP-2 by gelatin zymography. As shown in Figure 1, highest MMP-2 activity was associated with the low-density cultures, while activity gradually decreased with increasing cell density and was almost undetectable in the confluent cultures (Fig. 1, lanes 3–7). Similar results were obtained when cells were seeded at the same cell density (5×10^4 cells/dish) and allowed to grow, with the cell-bound MMP-2 being analyzed each day up to confluence (Fig. 1, D1–D4). Highest MMP-2 activity was detected one day after plating, whereas little or no activity was detected at confluence. MDA-MB-231 cells do not produce MMP-2 (Emonard *et al.*, 1992), and no MMP-2 activity could be detected either in the conditioned medium or in cell lysate (Fig. 1, lanes 1 and 2), whatever the cell density. In addition, Northern-blot analysis was negative for MMP-2 mRNA (not shown). Thus, the cell-associated activity detected in the presence of added enzyme corresponds to the exogenous MMP-2 bound. Binding of MMP-2 to MDA-MB-231 cells did not result in zymogen activation, as determined by the presence of a single gelatinolytic band of 72 kDa. Analysis of the medium before and after incubation of the cells gave identical zymographic profiles of the MMP-2 (not shown), confirming that no activation took place during incubation with the cells.

To rule out the possibility that the reduced association of MMP-2 with the surface of confluent MDA-MB-231 cells was due to the secretion of soluble factors, such as TIMP-2, that could prevent MMP-2 binding, low-density cultures were incubated with exogenous MMP-2 in the presence of serum-free conditioned medium obtained from confluent cultures. In these conditions, no differences in MMP-2 binding was observed, as determined by the intensity of gelatinolytic bands in zymograms (data not shown). When the detergent phase of the Triton X-114 lysates was analyzed, the bands obtained were barely detectable, but gave the same pattern as that obtained with the aqueous phase, suggesting that the exogenous MMP-2 bound to the cell surface dissociates in the presence of the 2% Triton X-114 used to lyse the cells (data not shown).

Cell-density-dependent binding of MMP-2 to other breast-carcinoma cells

In order to find out whether the regulation of MMP-2 binding by density observed with MDA-MB-231 can be found with other cells, 4 different breast-carcinoma cell lines, MDA-MB-435, ZR-75-1, T-47D and MCF-7, were compared with MDA-MB-231 for their capacity to bind MMP-2 as a function of density in culture. Figure 2 demonstrates that, in all cells examined, the binding of exogenous MMP-2 to the cells was profoundly influenced by the density of the culture, with much greater binding to sparse cultures.

Flow cytometry and immunocytochemical analysis of MMP-2 binding

To further analyze the binding of MMP-2 to low- and high-density cultures of MDA-MB-231 cells, we carried out flow-cytometry analysis of cells using a MAbs, CA-801, to MMP-2. MDA-MB-231 cells from sparse and confluent cultures were allowed to bind MMP-2, then washed and dislodged from the plates by scraping in PBS. Binding of cell-surface MMP-2 was monitored, using fixed non-permeabilized cells, with CA-801 and a FITC-labeled secondary antibody to mouse IgG. As shown in Figure 3, sparse cultures incubated with CA-801 elicited a 20% shift in cell-fluorescence intensity when compared with cells incubated with non-immune mouse IgG. In contrast, MDA-MB-231 cells obtained from confluent cultures and incubated with equal amounts of MMP-2 showed much lower fluorescence intensity, and only a 0.7% shift over untreated cells. The presence of MMP-2 on the surface of MDA-MB-231 cells at low cell density was demonstrated by immunocytochemical analysis on fixed

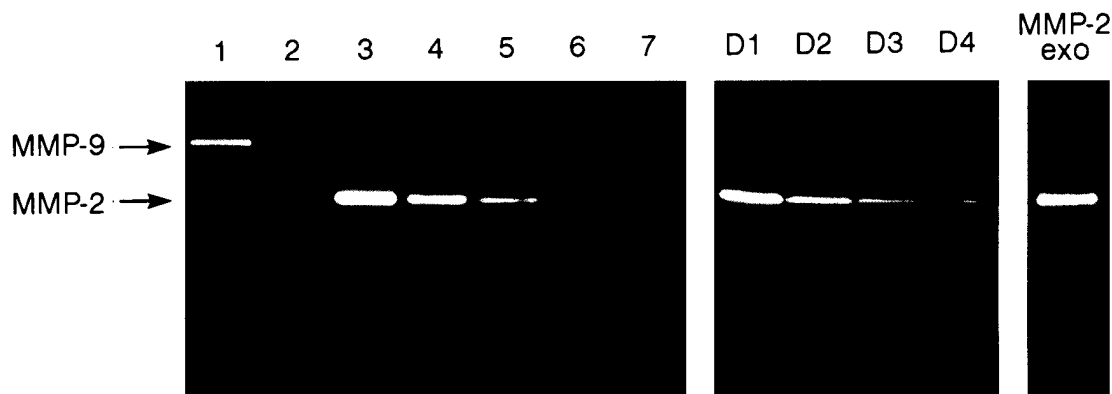


FIGURE 1 – Zymographic analysis of cell-associated MMP-2 in cultures of MDA-MB-231 cells of different density. Two methods were used to obtain different density: MDA-MB-231 cells were seeded at 5 different densities and lanes 3 to 7 correspond to cell densities of 5×10^4 , or 10^5 , 2×10^5 , 4×10^5 , 1×10^6 cells per 35-mm dish respectively. Increasing cell density was also obtained by seeding cells at the same density (5×10^4 cells per 35-mm dish) and allowing the cells to proliferate. Lanes D1 to D4 correspond to days in culture before analysis, where confluence was reached at Day 4. After incubation with 4 nM exogenous recombinant MMP-2 in serum-free DMEM, followed by 3 washes and lysis with 2% Triton X-114, aliquots of the aqueous fractions containing 10 µg protein were analyzed. In lane 1, 20 µl of conditioned media obtained after 24-hr incubation with serum-free DMEM were analyzed, showing that MDA-MB-231 secrete only MMP-9; negative control of sparse cells (10^5 per 35-mm dish), incubated without exogenous MMP-2 and analyzed as above, is shown in lane 2.

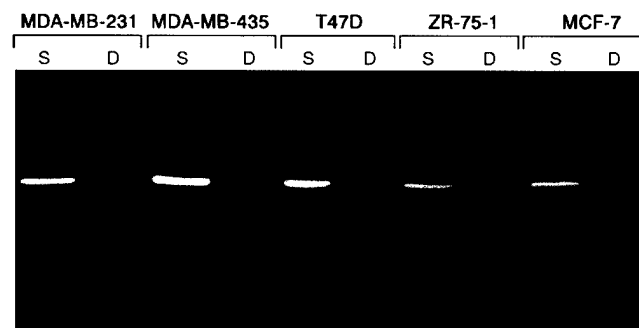


FIGURE 2 – MMP-2 binding to sparse and confluent cultures of different breast-carcinoma cells. MDA-MB-231, MDA-MB-435, T47D, ZR-75-1 and MCF-7 were seeded into 35-mm dishes at 2 different densities: 5×10^4 cells/dish (S sparse) and 4×10^5 cells/dish (D, dense), and allowed to attach in complete DMEM. After incubation with 4 nM exogenous recombinant MMP-2 in serum-free DMEM, aliquots of the aqueous fractions of Triton X-114 lysates containing 10 μ g protein were analyzed by gelatin zymography.

non-permeabilized cells, as described in “Material and Methods”. Some occasional staining was observed in confluent cultures; however, individual cells in sparse cultures were strongly stained (Fig. 4).

Binding of active MMP-2 and effect of TIMP-2 on MMP-2 binding to sparse cultures of MDA-MB-231 cells

Low-density cultures of MDA-MB-231 cells, exhibiting the greatest MMP-2-binding capacity, were used to examine whether the surface association of the exogenous latent MMP-2 was affected by activation of the pro-enzyme form. To this end, the latent enzyme was activated with APMA, which resulted in generation of the 62- and 45-kDa active species, as reported (Fridman *et al.*, 1995) (Fig. 5). As shown in the zymogram of Figure 5, the 62-kDa active form of MMP-2 was readily identified in the aqueous phase of the cells consistent with cell-surface binding (Fig. 5, lane 5). In contrast, the 45-kDa active species was not detected. We next examined the effect of different concentrations of TIMP-2 on the association of latent or active MMP-2 to sparse cultures of MDA-MB-231 cells. Cells were pre-incubated with 2, 4 or 10 nM TIMP-2 for 30 min, then 4 nM of either latent or activated MMP-2 were added. The cell-surface association of the enzymes was then examined by gelatin zymography. These studies showed that the presence of TIMP-2 had no significant effects on the association of latent MMP-2 (Fig. 5, lanes 1–4) but greatly inhibited the association of the active enzyme (Fig. 5, lanes 5–8). In addition, zymograms of confluent cultures that received MMP-2 with TIMP-2 showed no differences between cells that received enzyme alone and cells that received enzyme and TIMP-2, suggesting that the inhibitor did not increase the association of MMP-2 to high-density cultures (data not shown).

Effect of cell density on MT1-MMP, TIMP-2 and α v-integrin expression in MDA-MB-231 cells

Earlier studies have demonstrated that MMP-2 binding to the cell surface is mediated by an MT1-MMP/TIMP-2 complex (Strongin *et al.*, 1995), and also suggested that α v β 3 integrin may also play a role in the cell-surface association of MMP-2 (Brooks *et al.*, 1996). We therefore examined the expression of MT1-MMP, TIMP-2 (Fig. 6) and α v integrin (Fig. 7) in MDA-MB-231 cells, to determine whether the level of expression of these proteins was dependent on cell density. As shown in Figure 6a, MDA-MB-231 cells produce the 4.5-kb MT1-MMP mRNA transcript, and its level of expression in confluent cultures (Fig. 6a, lane 1) was 2.9-fold higher than in cells obtained from sparse cultures (Fig. 6a, lane 2). Since MT1-MMP is a plasma-membrane-bound enzyme, we examined the expression of MT1-MMP by immunoblot analysis of

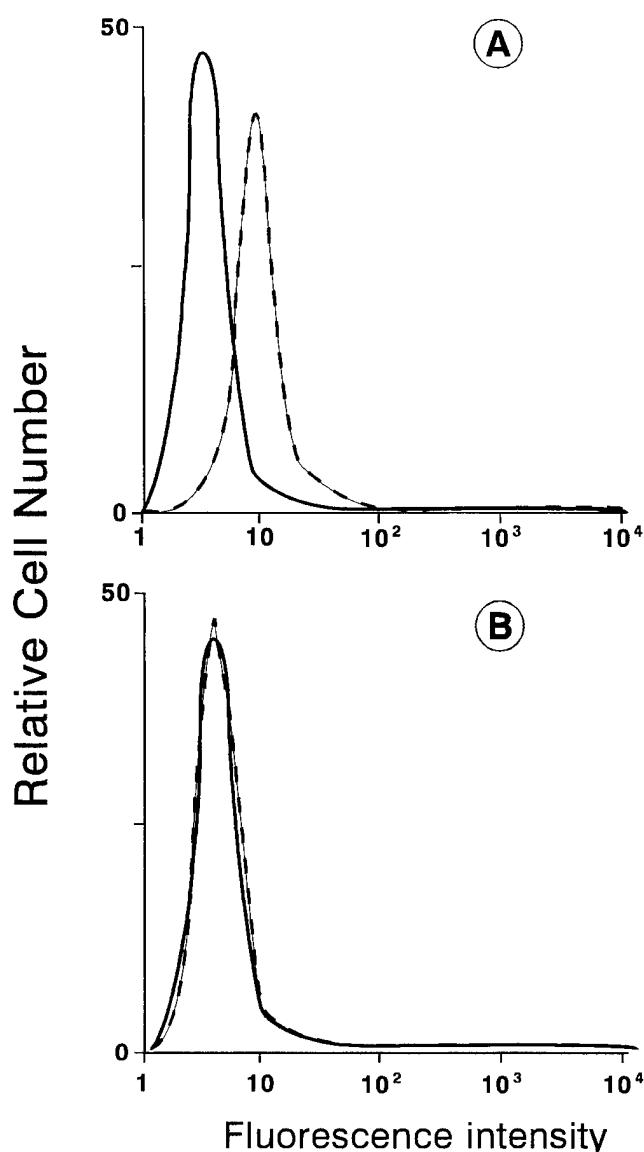


FIGURE 3 – Flow-cytometric analysis of the binding of MMP-2 to sparse and confluent MDA-MB-231 cells. Sparse (a) and confluent (b) cultures of MDA-MB-231 cells were prepared for flow cytometry as described in “Material and Methods”. Cells were incubated with 10 μ g/ml of either non-immune IgG (continuous line) or an anti-MMP-2 MAb (broken line).

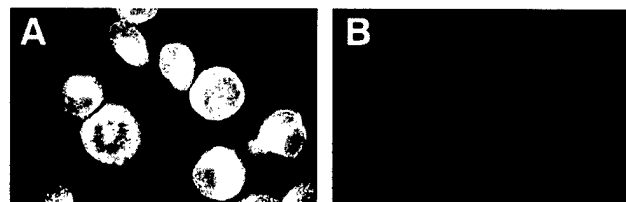


FIGURE 4 – Cell-surface-associated MMP-2 visualized by immunofluorescence. Cell cultures at Day 1 (a) and at confluence (b).

the detergent phase after Triton-X-114 phase separation of sparse and confluent cells, as described in “Material and Methods”. As shown in Figure 5b, confluent cultures (Fig. 6b, lane 1) exhibited a higher amount of MT1-MMP protein compared with the sparse cultures (Fig. 6b, lane 2), consistent with the results of the Northern

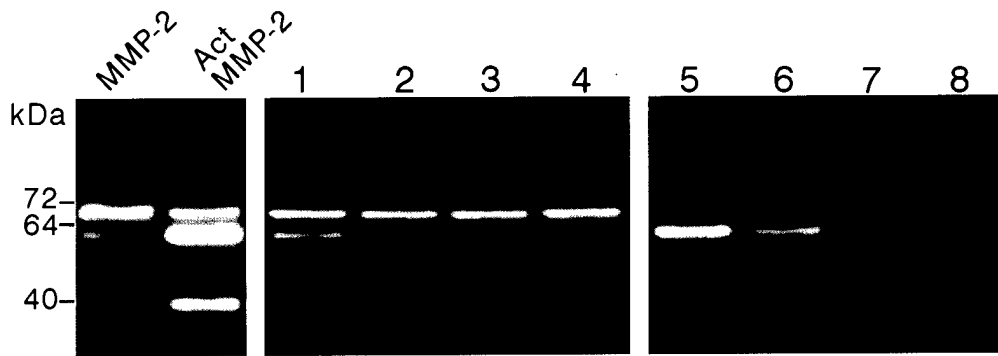


FIGURE 5 – Effect of TIMP-2 on the binding of latent and active forms of MMP-2 to sparse MDA-MB-231 cells by zymographic analysis. Activated MMP-2 was obtained as described in “Material and Methods” by incubating pro-MMP-2 with 1 mM APMA for 1 hr at room temperature, followed by removal of APMA by column chromatography. Cells were incubated with 4 nM of either latent (lane 1) or activated (lane 5) MMP-2; lanes 2,3,4, cells were pre-incubated with 2, 4 or 10 nM TIMP-2, respectively, for 30 min before the addition of 4 nM latent MMP-2; lanes 6,7,8, cells were pre-incubated with 2, 4 or 10 nM TIMP-2, respectively, for 30 min before the addition of 4 nM activated MMP-2. Binding of the enzymes was analyzed by gelatin zymography of the aqueous fraction of the Triton X-114 lysates; 2 ng of the latent (MMP-2) and activated (act MMP-2) enzymes are shown as standards.

blots. Expression of TIMP-2 mRNA (Fig. 6c) showed a transcript of 3.5 kb that was 2.5-fold higher in the confluent than in the sparse cells (Fig. 6c, lanes 1 and 2 respectively). Consistent with the results in the Northern blots, the media of confluent MDA-MB-231 cells contained higher amounts of TIMP-2 (Fig. 6d).

Detection of α_v integrin on MDA-MB-231 cells by immunoblot analysis was dependent on the protocol used to obtain the sparse and confluent cells. When cells were harvested 1 day (sparse) or 4 days (confluent) after seeding, α_v (145–150 kDa) levels were higher in the confluent cultures (Fig. 7a, lane 1). In contrast, when the cells were dislodged by trypsinization and then seeded at low and high densities and harvested the following day, α_v could not be detected in either culture (Fig. 7b). Nevertheless, these studies demonstrated an inverse correlation between expression of α_v integrin and MMP-2 cell-surface association in sparse and confluent cultures of MDA-MB-231 cells.

DISCUSSION

Tumor-cell invasion usually occurs in specific areas of the tumor mass, and involves the detachment and invasion of a limited number of malignant cells closely associated with the tumor-stroma interface. This heterogeneity in the invasive abilities within the tumor population is thought to be related to the expression of malignant properties on certain cells from the tumor mass. However, the mechanism regulating the differential expression of invasive abilities in breast-cancer cells is unknown. Since breast-carcinoma cells usually invade in small clusters that detach from the main tumor mass, we wished to determine whether cell-surface binding of MMP-2 may be regulated by cell density in MDA-MB-231 cells, a metastatic breast-cancer cell line (Price *et al.*, 1990). Cultured MDA-MB-231 cells do not secrete detectable levels of MMP-2, as determined by gelatin zymography. However, we have shown that addition of exogenous MMP-2 to these cells results in cell-surface association of the enzyme that is dependent on the density of the culture. High cell density, obtained either by plating increasing number of cells or by allowing cells to proliferate for various time periods, was associated with a significant decrease in the amount of exogenous MMP-2 that was bound to the cells, with almost no detectable enzyme bound to confluent cells. In contrast, MDA-MB-231 cells from sparse cultures exhibited enhanced binding of MMP-2, as demonstrated by 3 different methods. This density-dependent regulation of MMP-2 binding was also observed with other breast-carcinoma cells. Binding of MMP-2, in these conditions, did not result in enzyme activation, suggesting that other factors are required to induce activation. However, we observed that the activated 62-kDa form of MMP-2 also bound to

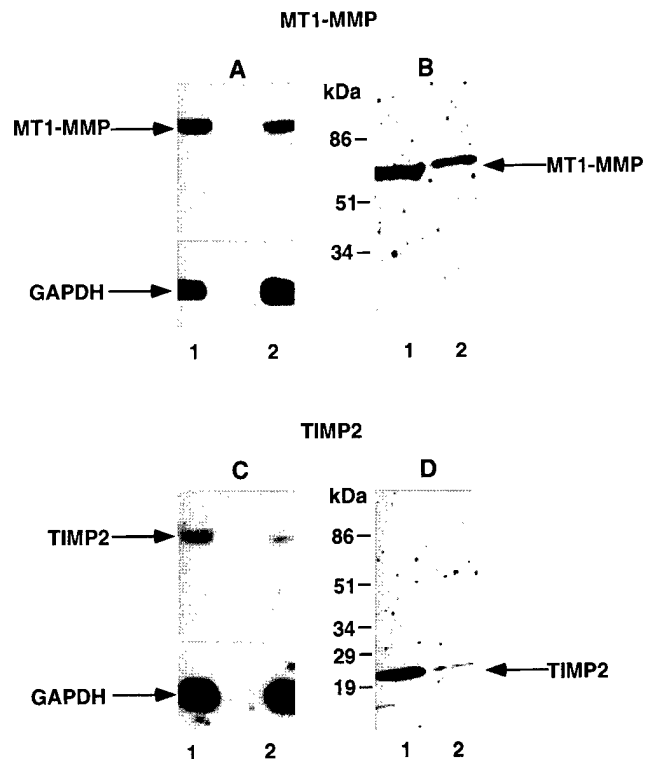


FIGURE 6 – Expression of MT1-MMP and TIMP-2 in confluent and sparse MDA-MB-231 cells. Northern-blot (a,c) analysis of total RNA isolated from confluent (lane 1) and sparse (lane 2) cultures of MDA-MB-231 cells. Equal amounts (20 μ g) of RNA were loaded on 1% agarose gels, blotted to nylon membrane and probed with either an MT1-MMP (a) or a TIMP-2 (c) cDNA probe. RNA loading was normalized using the signal obtained with a GAPDH cDNA probe. Immunoblot analysis of MT1-MMP (b) was carried out from confluent (lane 1) or sparse (lane 2) cultures of MDA-MB-231 cells after extraction with Triton X-114, as described in “Material and Methods”. Immunoblot analysis of TIMP-2 (d) expression was determined in the serum-free conditioned media of confluent (lane 1) and sparse (lane 2) cultures of MDA-MB-231 cells. Equal protein amounts of the samples were subjected to 4 to 12% (MT1-MMP) or 8 to 16% (TIMP-2) SDS-PAGE in reducing conditions and blotted to a nitrocellulose membrane. Detection of the antigens in the blots was performed using the ECL detection system (Pierce) according to the manufacturer’s instructions.

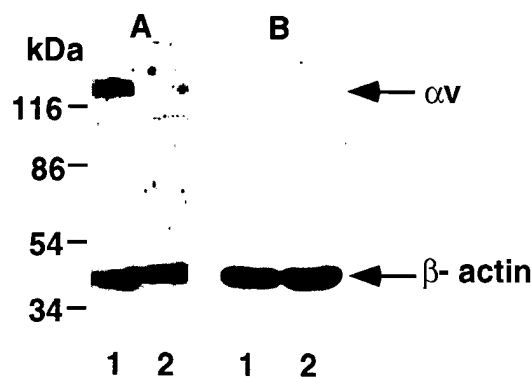


FIGURE 7 – Expression of αv in confluent and sparse MDA-MB-231 cells. Confluent (lane 1) or sparse (lane 2) cultures of MDA-MB-231 cells were obtained according to protocol 2 (a) or protocol 1 (b) as described in “Material and Methods”. Cells were lysed with lysis buffer, subjected to 4 to 12% SDS-PAGE in reducing conditions, then transferred to nitrocellulose. The blot was first developed with an anti- αv MAb (Chemicon 1960), then developed for the detection of β -actin with an anti- β -actin antibody as a control for loading. Detection of the antigens was carried out with the ECL detection system.

sparse cultures of MDA-MB-231 cells. Exposure of breast-carcinoma cell lines to concanavalin A (ConA) induces activation of MMP-2, a process shown to be mediated by MT1-MMP (Yu *et al.*, 1995). The results presented here suggest that binding of MMP-2 is independent from enzyme activation, and may not be mediated by enhanced expression of MT1-MMP.

In fact, confluent cultures of MDA-MB-231 cells showed higher levels of MT1-MMP and TIMP-2 expression, at the mRNA and the protein level, when compared with cells obtained from sparse cultures. Thus these proteins were unlikely to be the limiting factors for the low cell-surface association of MMP-2 in high-density cultures. The lack of correlation between levels of MT1-MMP expression and activation of MMP-2 have been reported in other cell systems after treatment with ConA or phorbol ester, consistent with the need of an additional co-operating factor(s). Here we show that enhanced binding of MMP-2 to the cell surface is not directly correlated with the level of MT1-MMP expression, again suggesting that other factor(s) may be involved in the binding of MMP-2 to the surface of MDA-MB-231 cells. It has been proposed that cell-surface binding of MMP-2 is mediated by an MT1-MMP/TIMP-2, with the latter acting as a cell-surface receptor for MMP-2 (Strongin *et al.*, 1995). However, TIMP-2 levels in sparse and confluent cells did not correlate with MMP-2 binding. In addition, the conditioned media of confluent MDA-MB-231 cells containing high levels of TIMP-2 had no effect on the binding of MMP-2 to the cells in sparse cultures, ruling out the possibility that TIMP-2 or any other soluble factor is responsible for decreased binding of MMP-2 to the confluent cells. Consistently, pre-

incubation of confluent MDA-MB-231 cells with exogenous TIMP-2 had no effect on MMP-2 binding. These results suggest that neither MT1-MMP nor TIMP-2 plays a direct role in the density regulation of MMP-2 binding to MDA-MB-231. It should be mentioned that, although MT1-MMP is expressed by breast-carcinoma cells *in vitro*, its expression *in vivo*, as determined by *in situ* hybridization studies, is confined to the stroma cells surrounding the invasive breast-cancer cells (Okada *et al.*, 1995). Likewise, TIMP-2 mRNA (Poulsom *et al.*, 1993) and protein (Visscher *et al.*, 1994) were found to be localized mostly in tumor stromal fibroblasts. In contrast, MMP-2 protein was consistently found on breast-cancer cells (Hoyhtya *et al.*, 1994; Visscher *et al.*, 1994). Thus, the role of MT1-MMP/TIMP-2 in the localization of MMP-2 on the cell surface of invasive breast-cancer cells remains unclear.

Integrin $\alpha v \beta 3$ may also be involved in the association of MMP-2 to the cell surface of melanoma cells (Brooks *et al.*, 1996). This association was shown to be mediated by the C-terminal domain of latent MMP-2. The role of integrin $\alpha v \beta 3$ in the cell-surface association of MMP-2 in breast-carcinoma cells has yet to be established, although our data indicate that in MDA-MB-231 cells the level of αv expression inversely correlated with MMP-2 binding since, in spite of the high levels of αv in the confluent cells, binding of MMP-2 was minimal. Thus the data presented here, although not ruling out a role for MT1-MMP, TIMP-2 and $\alpha v \beta 3$ in the cell-surface association of MMP-2 to MDA-MB-231 cells, are consistent with a complex mechanism of MMP-2 binding which is independent of the level of expression of these proteins but is regulated by cell density. The mechanism by which cell density regulates surface binding of MMP-2 is not understood; however, effects of cell density in MMP regulation have been observed in other studies. For example, the media of sparse cultures of astrocytoma cells contained more collagen-type-IV degrading activity than confluent cultures (Tamaki *et al.*, 1996), and epidermoid-carcinoma cells secreted MMP-9 in response to cytokines and growth factors only when the cells were in sparse cultures (Xie *et al.*, 1994). It was concluded that it was important for tumor cells to be located in areas of lower cell density in order to maximize the production and activity of gelatinases. Consistent with these findings, our data show that in low-density cultures of breast-carcinoma cells, MMP-2 utilization by the tumor cells is up-regulated at the level of MMP-2 binding to the cell surface. This increment in binding would concentrate MMP-2 at the migration front of the cell. By up-regulating MMP-2 binding on their surface, the migrating tumor cells can efficiently exploit MMP-2 secreted by connective tissue cells or stored in the extracellular matrix (Menashi *et al.*, 1995). This is consistent with immunohistochemical studies showing intense pericellular staining of invasive breast-cancer cells with antibodies to MMP-2 in sections of invasive breast carcinomas (Visscher *et al.*, 1994), while the enzyme mRNA is produced by stromal cells, as determined by *in situ* hybridization (Poulsom *et al.*, 1993). These findings and our results suggest that, *in vivo*, motile tumor cells could acquire a greater capacity to bind MMP-2 and consequently to degrade the extracellular matrix, a process crucial to their continued invasion.

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High Affinity Binding of Latent Matrix Metalloproteinase-9 to the $\alpha 2(\text{IV})$ Chain of Collagen IV*

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Matthew W. Olson^{‡§}, Marta Toth^{‡§}, David C. Gervasi[‡], Yoshikazu Sado[¶], Yoshifumi Ninomiya^{||}, and Rafael Fridman^{‡**}

From the [‡]Department of Pathology and the Karmanos Cancer Institute, Wayne State University, Detroit, Michigan 48201, the [¶]Division of Immunology Shigei Medical Research Institute 2117 Yamada, Okayama 701-02, Japan, and the ^{||}Department of Molecular Biology and Biochemistry, Okayama University Medical School, Shikata-cho, Okayama 700, Japan

Association of matrix metalloproteinases (MMPs) with the cell surface and with areas of cell-matrix contacts is critical for extracellular matrix degradation. Previously, we showed the surface association of pro-MMP-9 in human breast epithelial MCF10A cells. Here, we have characterized the binding parameters of pro-MMP-9 and show that the enzyme binds with high affinity ($K_d \sim 22$ nM) to MCF10A cells and other cell lines. Binding of pro-MMP-9 to MCF10A cells does not result in zymogen activation and is not followed by ligand internalization, even after complex formation with tissue inhibitor of metalloproteinase-1 (TIMP-1). A 190-kDa cell surface protein was identified by ligand blot analysis and affinity purification with immobilized pro-MMP-9. Microsequencing and immunoblot analysis revealed that the 190-kDa protein is the $\alpha 2(\text{IV})$ chain of collagen IV. Specific pro-MMP-9 surface binding was competed with purified $\alpha 2(\text{IV})$ and was significantly reduced after treatment of the cells with active MMP-9 before the binding assay since $\alpha 2(\text{IV})$ is hydrolyzed by MMP-9. A pro-MMP-9-TIMP-1 complex and MMP-9 bind to $\alpha 2(\text{IV})$, suggesting that neither the C-terminal nor the N-terminal domain of the enzyme is directly involved in $\alpha 2(\text{IV})$ binding. The closely related pro-MMP-2 exhibits a weaker affinity for $\alpha 2(\text{IV})$ compared with that of pro-MMP-9, suggesting that sites other than the gelatin-binding domain may be involved in the binding of $\alpha 2(\text{IV})$ to pro-MMP-9. Although pro-MMP-9 forms a complex with $\alpha 2(\text{IV})$, the proenzyme does not bind to triple-helical collagen IV. These studies suggest a unique interaction between pro-MMP-9 and $\alpha 2(\text{IV})$ that may play a role in targeting the zymogen to cell-matrix contacts and in the degradation of the collagen IV network.

proteolytic enzymes closely associated with discrete areas of cell-matrix contacts. These include the plasminogen/plasmin system (1), cathepsins (2), and MMPs (3–6). The gelatinases A (MMP-2) and B (MMP-9) are two members of the MMP family that have been shown to play a central role in many normal and pathological conditions involving ECM degradation, including wound healing, angiogenesis, embryogenesis, arthritis, and tumor metastasis (3–6). Like other members of the MMP family, the gelatinases are produced in a latent form (pro-MMP)² that requires activation to become proteolytically active. Thus, activation is a critical step in the regulation of MMP-dependent proteolytic activity. Considerable evidence has associated the gelatinases with the ability of tumor cells to metastasize due to their ability to degrade basement membrane collagen IV and to their elevated expression in malignant tumors (5, 6). Previous studies have shown that tumor cells contain gelatinase activity in the plasma membranes consistent with cell surface association (7–10). We have shown a surface localization of both gelatinases in carcinoma cells of breast tumors (11, 12) and epithelial cells of fibrocystic breast disease (12), and others have shown the localization of pro-MMP-9 in the tumor basement membrane zone of skin tumors (13) colocalizing with collagen IV.³ In recent years, much information has been gained on the cell surface association of pro-MMP-2. It has been shown that pro-MMP-2 binds to the cell surface via membrane type 1-MMP (MT1-MMP), a subclass of MMPs bound to plasma membranes (14, 15). Interestingly, the binding of pro-MMP-2 to MT1-MMP requires the participation of tissue inhibitor of metalloproteinase-2 (TIMP-2) (14). The trimer complex allows for pro-MMP-2 activation on the cell surface, possibly by another MT1-MMP molecule. A later study suggested that the cell surface association of pro-MMP-2 can also occur through the binding of the C-terminal domain of the enzyme to integrin $\alpha_v\beta_3$ (16). Thus, several mechanisms may play a role in the surface localization of pro-MMP-2.

Pro-MMP-9 is structurally similar to pro-MMP-2, with both enzymes containing three tandem copies of a 58-amino acid residue fibronectin type II-like module (17, 18), known as the gelatin-binding domain, which plays a role in binding to extracellular matrix components (19–21). Furthermore, both latent enzymes can bind a TIMP molecule in the C-terminal domain of the zymogen with pro-MMP-9 binding to TIMP-1 (17) and pro-MMP-2 to TIMP-2 (4, 5). Pro-MMP-9 also contains a 54-amino acid proline-rich extension of unknown function that is

The degradation of ECM¹ components is partly achieved by

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§ Both authors contributed equally to this work.

** To whom all correspondence should be addressed: Dept. of Pathology, Wayne State University, 540 E. Canfield Ave., Detroit, MI 48201. Tel.: 313-577-1218; Fax: 313-577-8180; E-mail: rfridman@med.wayne.edu.

¹ The abbreviations used are: ECM, extracellular matrix; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; MT1-MMP, membrane type 1-MMP; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; FBS, fetal bovine serum; PMSF, phenylmethylsulfonyl fluoride; HRP, horseradish perox-

idase; EHS, Engelbreth-Holm-Swarm; ECL, enhanced chemiluminescence; mAb, monoclonal antibody; RVE, rat vascular endothelial.

² Latent enzyme will be referred to as "pro-MMP" and active enzyme as "MMP."

³ L. Coussens, personal communication.

similar to the $\alpha 2(V)$ chain of collagen V and in addition is a glycosylated enzyme (17). In contrast to pro-MMP-2, little is known about the interactions of pro-MMP-9 with the cell surface, yet pro-MMP-9 has been found to be present on the cell surface. Recent studies by Partridge *et al.* (22) showed that pro-MMP-9 is present in the plasma membrane and focal contacts of cultured endothelial cells. Pro-MMP-9 was also detected in the plasma membranes of human fibrosarcoma HT1080 cells (23), and the enzyme could be activated by a plasmin-dependent mechanism (24). We have recently shown that, upon induction with 12-*O*-tetradecanoylphorbol-13-acetate, pro-MMP-9 binds to the surface of human breast epithelial MCF10A cells (25). In this report, we further examined the binding of pro-MMP-9 to a variety of cell lines and demonstrated the existence of a single high affinity ($K_d \sim 22$ nM) binding site. Using immobilized pro-MMP-9, ligand blot analysis and co-immunoprecipitation experiments with surface biotinylated cells, we have identified the $\alpha 2(IV)$ chain of collagen IV as the major pro-MMP-9-binding protein. These studies provide novel evidence on the interactions of pro-MMP-9 with $\alpha 2(IV)$ that may play a role in the localization of zymogen at cell-matrix contacts and degradation of basement membranes.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant Gelatinases and TIMP-1—Human recombinant pro-MMP-9, pro-MMP-2, and TIMP-1 were all expressed in HeLa cells using a recombinant vaccinia virus expression system and purified to homogeneity as described previously (26, 27). To obtain ^{35}S -pro-MMP-9 and ^{35}S -pro-MMP-2, expression of recombinant enzymes in infected HeLa cells was carried out in the presence of 15 $\mu\text{Ci/ml}$ [^{35}S]methionine. The specific activities of ^{35}S -pro-MMP-2 and ^{35}S -pro-MMP-9 were 0.0159 $\mu\text{Ci/pmol}$ and 0.0106 $\mu\text{Ci/pmol}$, respectively.

Antibodies—The monoclonal antibodies to human pro-MMP-9 (CA-209) and to pro-MMP-2 (CA-801 and CA-805) have been described previously (11, 12, 25, 28). An anti-pro-MMP-9 rabbit polyclonal antibody (pAB109) raised against a synthetic peptide (APRQRQSTLVLT-PGDLRT) from the N-terminal domain of human pro-MMP-9 was a generous gift from Dr. Stetler-Stevenson (National Cancer Institute, National Institutes of Health, Bethesda, MD) (25). A polyclonal antibody against human TIMP-1 was raised in rabbits and reacts with TIMP-1 but not with TIMP-2 (25). Anti-pro-MMP-2 polyclonal antibodies were raised in rabbits against human recombinant pro-MMP-2 (29). Mouse monoclonal antibodies (mAbs) against human TIMP-1 (IM 322) were purchased from Calbiochem (San Diego, CA). Chain-specific rat mAbs to human collagen IV were prepared as described previously (30). A mAb to the $\alpha 2(IV)$ chain of human collagen IV (mAb 1910) was purchased from Chemicon (Temecula, CA).

Iodination of Pro-MMP-9 and TIMP-1—Pro-MMP-9 or TIMP-1 (50–100 μg) in 100 μl of collagenase buffer (50 mM Tris-HCl (pH 7.5), 5 mM CaCl_2 , 150 mM NaCl, and 0.02% Brij-35) were placed in a vial coated with IODOGEN (Pierce) as described by the manufacturer and allowed to incubate for 1 min at 25 °C. Na^{125}I (500 μCi) was added to each vial, and the iodination reaction was allowed to continue for 3 min at 25 °C. The reaction was quenched by the addition of 200 μg (100 μl) of bovine serum albumin (BSA) and 2 mM NaI. Unincorporated Na^{125}I was removed by a 1-ml Sephadex G-25 (fine) column equilibrated with collagenase buffer. The specific activity of ^{125}I -pro-MMP-9 and ^{125}I -TIMP-1 was determined after trichloroacetic acid precipitation and quantitation by densitometry of the proteins in Coomassie Blue-stained SDS-polyacrylamide gels relative to a standard curve of unlabeled purified proteins. Typically, the specific activities of ^{125}I -TIMP-1 and ^{125}I -pro-MMP-9 were 0.049 $\mu\text{Ci/pmol}$ and 0.322 $\mu\text{Ci/pmol}$, respectively. No detectable autocatalytic/degradation forms of MMP-9 were observed in the iodinated enzyme as determined by both gelatin-zymography and autoradiography.

Cells—Human immortalized breast epithelial MCF10A cells (31) were grown as described previously (25). MDA-MB-231 breast cancer cells and PC3 prostate cancer cells were provided by Dr. Fred Miller (Karmanos Cancer Institute, Detroit, MI) and grown in DMEM supplemented with 10% fetal bovine serum (FBS) and RPMI 1640 medium supplemented with 10% FBS, respectively. Human HT1080 fibrosarcoma (CCL-121) cells were obtained from the American Type Culture Collection (Rockville, MD) and grown in DMEM supplemented with 10% FBS. Rat vascular endothelial (RVE) cells derived from brain

capillaries (32) and mouse lung microvascular endothelial (CD3) cells were provided by Dr. Diglio (Department of Pathology, Wayne State University) and grown in DMEM supplemented with 10% FBS.

Binding Assays—Cells (MCF10A, MDA-MB-231, PC3) grown to 80% confluence (3–5 days after seeding) in 12-well (22-mm) plates were rinsed with PBS and incubated (15 min at 4 °C) with cold binding medium (25 mM Hepes (pH 7.5) with 0.5% BSA in DMEM). The medium was aspirated, and various concentrations (1–40 nM) of ^{125}I -pro-MMP-9 diluted in binding medium were added to each well (300 μl) in the presence or absence of 80-fold excess unlabeled pro-MMP-9. After a 45-min incubation at 4 °C, the medium was aspirated and the cells were washed three times with cold PBS containing 0.1% BSA. The cells were lysed with 0.5 ml/well of 0.5 M NaOH for determination of radioactive counts in a γ counter (Packard model 5650), and the results were expressed as the mean of the values obtained from triplicate samples. The number of cells in each well was determined in quadruplicate wells. Time-course experiments were similarly done, except that the concentration of ^{125}I -pro-MMP-9 for each well was kept constant at 18 nM and the cells were harvested after various times at 4 °C. The nonspecific binding of ^{125}I -pro-MMP-9 was determined in the presence of 80-fold excess unlabeled pro-MMP-9. Typically, specific binding represented an average of approximately 20–30% of total bound ^{125}I -pro-MMP-9. The association rate constant (k_{on}) of pro-MMP-9 was determined from the time-course experiment following logarithmic transformation of the amount of specifically bound ^{125}I -pro-MMP-9 versus time. Binding of ^{125}I -pro-MMP-9 to the cell surface follows a second-order binding isotherm. Thus, the k_{on} is determined from the slope of a line plotted as $\ln([LReq]/([LReq] - [LR]))$ versus time as described previously (33), where $LReq$ is the quantity of ^{125}I -pro-MMP-9 bound at 120 min and LR is the quantity of ^{125}I -pro-MMP-9 bound at the times 0, 2, 15, and 30 min. The slope of this line was determined by linear regression analysis using Microsoft Excel™, and the error represents the standard deviation of the slope. The equilibrium binding constant (K_d) and the number of binding sites per cell were determined by nonlinear curve-fitting analysis using the GraphPad Prism™ software version 2.0 and by Scatchard analysis. The slope and the intercept from the Scatchard analysis were determined by linear regression analysis using Microsoft Excel™. For the latter, the error represents the standard deviation of the slope and intercept. For HT1080 and RVE cells, binding was performed with ^{125}I -pro-MMP-9 (3.6 nM) in the presence and absence of 80-fold excess unlabeled pro-MMP-9 for 45 min at 4 °C and the amount of specific ligand bound (fmol/cells) was determined. Competition of ^{125}I -pro-MMP-9 binding with pro-MMP-2 was carried out in a ligand binding assay as described above, except that 80-fold excess unlabeled pro-MMP-2 was used instead of unlabeled pro-MMP-9.

Cellular Distribution of Bound ^{125}I -Pro-MMP-9—MCF10A cells were incubated with cold binding medium and then incubated with 18 nM/well of ^{125}I -pro-MMP-9 in triplicate wells for 45 min at 4 °C. The medium was aspirated, and the cells were washed four times with cold binding medium. Each well then received 0.5 ml of prewarmed (37 °C) binding medium, and the plates were incubated at 37 °C for various times. At each time point, the medium was recovered and the cells were washed with PBS, followed by the addition of 0.5 ml/well of 0.25% Pronase E (Sigma) in PBS. The cells were incubated at 4 °C for 30 min, and the monolayer was dislodged by gentle pipetting and transferred to a microfuge tube. The samples were centrifuged for 5 min at 2000 $\times g$, and the supernatant (cell surface-bound fraction) was transferred to a new tube. The pellet (internalized fraction) was washed once with PBS and then resuspended in 0.5 ml of PBS. The radioactivity of the three fractions, in triplicate, was measured in a γ counter. Internalization studies in the presence of TIMP-1 were performed similarly, except that the cells were incubated with a ^{125}I -pro-MMP-9-TIMP-1 complex that was previously formed by incubating ^{125}I -pro-MMP-9 with TIMP-1 for 30 min at 22 °C.

Competition of ^{125}I -Pro-MMP-9 Binding with Purified $\alpha 2(IV)$ — ^{125}I -Pro-MMP-9 (3.6 nM) was incubated for 1.5 h at 4 °C with ~ 2 -fold molar excess of affinity-purified $\alpha 2(IV)$ at a final concentration of 7 nM in the presence of 5 mM EDTA. Binding assays with MCF10A cells were carried out as described above.

Binding of ^{125}I -Pro-MMP-9 to Cells Treated with MMP-9—MCF10A cells were incubated for 45 min at 37 °C in binding medium with 1.2 pmol/well (final concentration of 4 nM) of either purified 82-kDa active species of MMP-9 or MMP-9 that was previously incubated with TIMP-1 (2.4 pmol) for 30 min at 22 °C. After treatment, the cells were washed twice with cold binding medium and incubated for 15 min in binding medium at 4 °C. Binding assays were carried out as described above.

Coupling of Pro-MMP-9 to Affi-Gel 10—One milligram of recombi-

nant pro-MMP-9 in 50 mM Hepes (pH 7.5), 5 mM CaCl_2 , 150 mM NaCl, and 0.02% Brij-35 was allowed to bind to 1 ml of Affi-Gel 10 (Bio-Rad) for 5 h at 4 °C with rotation in the presence of 60 mM CaCl_2 . After coupling, the matrix (Affi-Gel 10-pro-MMP-9) received a 150- μl volume of 1 M ethanolamine (pH 8) and incubated with rotation for 1 h at 4 °C. The matrix was allowed to settle, and the supernatant was subjected to SDS-PAGE to determine the amount of uncoupled pro-MMP-9. The Affi-Gel 10-pro-MMP-9 matrix was washed four times with collagenase buffer. The immobilized pro-MMP-9 maintained its capability to bind TIMP-1, as determined by binding of ^{125}I -TIMP-1 compared with soluble enzyme.

Affinity Purification of the 190-kDa Protein—MCF10A cells were lysed with (0.8 ml/150-mm plate) ice-cold lysis buffer (25 mM Tris (pH 7.5), 100 mM NaCl, 1% Nonidet P-40, 10 $\mu\text{g}/\text{ml}$ aprotinin, 1 $\mu\text{g}/\text{ml}$ leupeptin, 5 mM benzamide, and 1 mM PMSF). After a centrifugation (20 min, 14,000 rpm), the supernatant was collected and incubated (4 °C) with Affi-Gel 10-pro-MMP-9 batchwise overnight, poured into a column (PolyprepTM, Bio-Rad), and the flow-through fraction collected. The matrix was washed with 20 ml of 25 mM Tris (pH 7.5), 500 mM NaCl, 0.1% Nonidet P-40, 2 mM PMSF, 5 mM benzamide, 10 $\mu\text{g}/\text{ml}$ leupeptin, and 10 $\mu\text{g}/\text{ml}$ aprotinin (wash 1), followed by 10 ml of the same buffer as described above but containing 150 mM NaCl (wash 2). The 190-kDa protein was eluted from the column with 4.5 ml of 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM CaCl_2 , 20% Me_2SO , 2 mM PMSF, 5 mM benzamide, 10 $\mu\text{g}/\text{ml}$ leupeptin, and 10 $\mu\text{g}/\text{ml}$ aprotinin. Three 1.5-ml fractions of eluate were collected. Forty microliters of the load, flow-through, wash 1, wash 2, and eluate fractions were analyzed by silver-stained SDS-PAGE (34) and ligand blot as described below. The protein concentrations of each column fraction were determined by the BCA protein assay (Pierce) reagent. The same procedure was used with lysates of surface biotinylated cells, except that 0.5–1 ml of lysates were incubated with 50 μl of Affi-Gel 10-pro-MMP-9 or uncoupled Affi-Gel 10 matrix. After binding, the matrix was washed with 1 ml of cold HNTG buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Nonidet P-40, 10% glycerol) followed by one wash in the same buffer containing 500 mM NaCl and three washes in HNTG buffer. After a brief centrifugation, the bound proteins were eluted with 40 μl of collagenase buffer with 10% Me_2SO and subjected to SDS-PAGE, blotting, and detection by streptavidin-HRP or ligand blot as described below.

Microsequencing—The affinity-purified 190-kDa protein was subjected to 7% SDS-PAGE under reducing conditions, followed by staining with 0.25% Coomassie Brilliant Blue. The band containing the 190-kDa protein was excised from the gel, and the pieces were washed three times (15 min each) with Millipore water. The gel slices were washed three times (5 min each) in 50% acetonitrile (Aldrich, HPLC grade), frozen in dry ice, and then sent to Dr. William Lane at the Harvard Microchemistry sequencing facility (Cambridge, MA).

Stokes Radius Determination—Affinity-purified $\alpha 2(\text{IV})$ (250 ng) was chromatographed on a Superose 6 (10/30) column (Amersham Pharmacia Biotech) equilibrated with 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, and 0.02% Brij-35 using an FPLC system at a flow rate of 0.15 ml/min and 0.3 ml fractions were collected. Samples (200 μl) from each fraction were trichloroacetic acid-precipitated (10% v/v) and analyzed for the presence of $\alpha 2(\text{IV})$ by SDS-PAGE under reducing conditions followed by ligand blot analysis. The column was calibrated with thyroglobulin, ferritin, catalase, aldolase, bovine serum albumin, ovalbumin, and chymotrypsin (Amersham Pharmacia Biotech).

Glycerol Gradient Sedimentation—Purified $\alpha 2(\text{IV})$ (500 ng) was layered onto two 15–30% glycerol gradients in 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.02% Brij-35, 2 mM PMSF, and 4 mM benzamide in polyallomer tubes. The tubes were then centrifuged (35,000 rpm for 54 h at 4 °C) in an SW 41 rotor. The fractions (0.2 ml) were collected and precipitated with trichloroacetic acid (10% v/v). The precipitates were subjected to SDS-PAGE under reducing conditions followed by ligand blot analysis as described below.

Cell Surface Biotinylation—Surface proteins were biotinylated with sulfo-NHS-biotin (Pierce) as described previously (25). The biotinylated cells were lysed with 2 ml/dish of ice-cold lysis buffer, the lysates incubated for 1 h on ice, and the supernatant collected after a 15-min centrifugation (13,000 $\times g$) at 4 °C. The supernatants were analyzed immediately for the presence of pro-MMP-9-binding proteins by pro-MMP-9-affinity chromatography or co-immunoprecipitation with pro-MMP-9 and detection with streptavidin-HRP as described below.

Preparation of Conditioned Medium—Confluent cultures of MCF10A cells were incubated with serum-free DMEM/F-12 (15 ml/150-mm dish) for 24 h at 37 °C. The medium was collected, centrifuged, and concentrated (6-fold) with a Centriprep-10 (Amicon). The medium was analyzed by immunoblot analysis as described above. In some experiments,

cells were grown in the presence of daily additions of ascorbate (75 $\mu\text{g}/\text{ml}$) and the conditioned medium was obtained as described.

Co-immunoprecipitation of $\alpha 2(\text{IV})$ with Pro-MMP-9—Lysates of surface-biotinylated or non-biotinylated cells in lysis buffer or samples of concentrated serum-free conditioned medium were incubated with 0.5–1 $\mu\text{g}/\text{ml}$ purified pro-MMP-9 for 1 h at 4 °C. In some experiments, the lysates received active MMP-9, pro-MMP-2, active MMP-2, or TIMP-1 in the presence or absence of 10 mM EDTA. Five micrograms of the appropriate antibody or control IgG were added for another 16-h incubation at 4 °C. Each sample received 25 μl of protein G-Sepharose 4 Fast Flow matrix (Amersham Pharmacia Biotech) and incubated for 3 h at 4 °C with continuous rocking. The matrix was washed with ice-cold HNTG buffer, followed by one wash in the same buffer supplemented with 500 mM NaCl and three additional washes with HNTG buffer. The samples were boiled in the presence of 20 μl of 2 \times Laemmli sample buffer (35) with reducing agent, and the supernatants were subjected to SDS-PAGE and the proteins transferred to a nitrocellulose membrane for immunoblot analysis or ligand blot analysis as described below.

Immunoblot and Ligand Blot Analysis—Samples resolved by SDS-PAGE were transferred to a BA-S 85 nitrocellulose membrane (Schleicher & Schuell). The membrane was blocked (12 h at 4 °C) with Blotto (3% BSA and 3% nonfat dry milk in 100 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.02% NaN_3) and washed twice with T-TBS (20 mM Tris (pH 7.5), 137 mM NaCl, and 0.1% Tween 20). For immunoblot analysis, the membranes were incubated with the appropriate primary antibody diluted in T-TBS containing 0.5% nonfat dry milk, washed three times with T-TBS and incubated with the HRP-conjugated secondary antibody. The immunodetection of the antigen was performed using ECL (Amersham Pharmacia Biotech) according to the manufacturer's instructions. For biotinylated samples, the membranes were incubated with streptavidin-HRP (Amersham Pharmacia Biotech) and developed by ECL. For ligand blot analysis, the membranes were incubated (1 h at 25 °C) with 1 $\mu\text{g}/\text{ml}$ pro-MMP-9 in T-TBS containing 0.5% nonfat dry milk and 1% Nonidet P-40, followed by three washes with T-TBS. After washing, the membranes were incubated (1 h at 25 °C) with anti-MMP-9 antibodies diluted in T-TBS containing 0.5% nonfat dry milk. After three washes in T-TBS, the membranes were incubated (1 h at 25 °C) with the HRP-conjugated secondary antibody in T-TBS, followed by three washes with T-TBS. The proteins were detected as described above.

Binding of Mouse-EHS Collagen IV to Immobilized Pro-MMP-9—EHS native collagen IV (a generous gift from Dr. Hynda Kleinman, National Institutes of Health, Bethesda, MD) was diluted (10 $\mu\text{g}/\text{ml}$) in lysis buffer (0.5 ml) and incubated (12 h at 4 °C) with 30 μl of Affi-Gel 10-pro-MMP-9 matrix. After incubation, the beads were centrifuged (13,000 $\times g$, 1 min at 4 °C) and the supernatant was collected (unbound fraction). The beads were washed with ice-cold HNTG buffer, twice with HNTG buffer containing 500 mM NaCl and a final wash in HNTG buffer. The beads were resuspended in 50 μl of 10% Me_2SO in collagenase buffer and mixed for 30 min at 4 °C, followed by a brief centrifugation to obtain the supernatant (bound fraction). The bound and unbound fractions were mixed with sample buffer under reducing conditions and subjected to SDS-PAGE, followed by ligand blot analysis as described above.

Determination of the Affinity of $\alpha 2(\text{IV})$ for Pro-MMP-2 and Pro-MMP-9—Affinity-purified $\alpha 2(\text{IV})$ (9.5 nM) was allowed to complex with varying concentrations of either ^{35}S -pro-MMP-2 (20–750 nM) or ^{35}S -pro-MMP-9 (2.5–250 nM) for 1 h at 4 °C. The samples were then subjected to gel filtration using a Superose 6 column (10/30) (Amersham Pharmacia Biotech) equilibrated with 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, and 0.02% Brij-35 at a flow rate of 0.2 ml/min, and 70 fractions (0.3 ml) were collected. Aliquots (0.2 ml) of each fraction were placed in 5 ml of scintillation fluid and counted to determine the amount (pmol) of enzyme bound. Complex formation was evaluated based on the relative inclusion volume of pro-MMP-2, pro-MMP-9, or $\alpha 2(\text{IV})$ alone or in complex. The dissociation constant (K_d) was determined from the binding profiles (picomoles of pro-MMP bound versus pro-MMP concentration) by extrapolating the point where 50% maximal binding was obtained.

RESULTS

Characteristics of ^{125}I -Pro-MMP-9 Binding to MCF10A Cells—Previously, we showed that exposure of MCF10A cells to 12-*O*-tetradecanoylphorbol-13-acetate results in the secretion of pro-MMP-9 that associates with the cell surface (25). To measure the binding of pro-MMP-9, we carried out binding

assays of radioiodinated enzyme to untreated MCF10A cells to avoid interference with the endogenously produced pro-MMP-9 (25). Fig. 1A shows time-dependent binding of ^{125}I -pro-MMP-9 to MCF10A cells at 4 °C. Logarithmic transformation of the data revealed a k_{on} value of $6.94 \pm 0.67 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ (Fig. 1A, inset). Incubation of the cells with increasing concentrations (1–40 nM) of ^{125}I -pro-MMP-9 in the presence of 80-fold excess of unlabeled enzyme demonstrated a specific saturable binding (Fig. 1B). Scatchard analysis revealed a K_d value of $2.16 \pm 0.16 \times 10^{-8} \text{ M}$ and 1.3×10^5 sites/cell (Fig. 1B, inset). Similar values were obtained after nonlinear curve-fitting analysis of the data ($K_d = 2.49 \pm 0.27 \times 10^{-8} \text{ M}$ and 1.4×10^5 sites/cell). We found no evidence of cell surface activation of ^{125}I -pro-MMP-9, as determined by SDS-PAGE followed by autoradiography or by gelatin zymography (data not shown).

Ligand binding assays were also performed with prostate carcinoma PC3 cells ($K_d = 2.62 \pm 0.56 \times 10^{-8} \text{ M}$ and 5.5×10^4 sites/cell) and breast carcinoma MDA-MB-231 cells ($K_d = 2.13 \pm 0.23 \times 10^{-8} \text{ M}$ and $1.08 \pm 0.1 \times 10^4$ sites/cell). In addition, similar k_{on} values ($\sim 6.7\text{--}8.1 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$) were obtained with both cell lines. We also measured the specific binding of ^{125}I -pro-MMP-9 to HT1080 and RVE cells and obtained the following values: HT1080 cells, 7 fmol/ 10^5 cells; RVE cells, 12.4 fmol/ 10^5 cells.

Binding specificity for pro-MMP-9 was determined in MCF10A cells using pro-MMP-2 as a competitor as described under "Experimental Procedures." These studies showed that pro-MMP-2 caused a 27% decrease in specific ^{125}I -pro-MMP-9 binding with 3.3 fmol/ 1.3×10^5 cells in the presence of unlabeled pro-MMP-2 compared with 4.5 fmol/ 1.3×10^5 cells with pro-MMP-9 alone. Thus, pro-MMP-2 slightly competes with pro-MMP-9 for the same binding site on MCF10A cells.

Cellular Distribution of ^{125}I -Pro-MMP-9 and a ^{125}I -Pro-MMP-9-TIMP-1 Complex after Binding to MCF10A Cells—We investigated the fate of pro-MMP-9 after binding using ligand internalization studies as described under "Experimental Procedures." Fig. 2A shows that the amount of ^{125}I -pro-MMP-9 associated with the cell pellet (internalized ligand) reached a maximum of 21% of the total ligand bound after 10 min of incubation at 37 °C. Later time points showed little or no change (<5%) in the radioactivity of the pellet fraction. Concurrently, after a 10-min incubation, $\sim 80\%$ of the total cell-associated radioactivity was recovered by Pronase E digestion (representing cell surface-bound enzyme). This fraction exhibited a decline in radioactivity consistent with dissociation of cell surface-bound ligand and its appearance in the supernatant fraction (Fig. 2A). SDS-PAGE analysis demonstrated that the radioiodinated enzyme in the supernatant fraction remained in the latent form at all times examined. In contrast, the internalized enzyme was degraded to low (≥ 20 kDa) molecular mass fragments (data not shown). Thus, binding of pro-MMP-9 to MCF10A cells is not followed by significant ligand internalization.

Binding studies with urokinase plasminogen activator demonstrated that binding of this activator to its receptor in the presence of plasminogen activator inhibitor-1 results in rapid internalization of the enzyme/inhibitor complex (36). To determine if a similar process could occur with pro-MMP-9, we examined the cellular partitioning of a ^{125}I -pro-MMP-9-TIMP-1 complex after binding to MCF10A cells as described above. These experiments showed no significant differences in the cellular distribution of the free (Fig. 2A) versus that of the complex (Fig. 2B) ligand after incubation at 37 °C for up to 2 h. Furthermore, the association of pro-MMP-9 with TIMP-1 did not alter the rate of ligand dissociation into the supernatant fraction as $>90\%$ of the radioactivity in this fraction was tri-

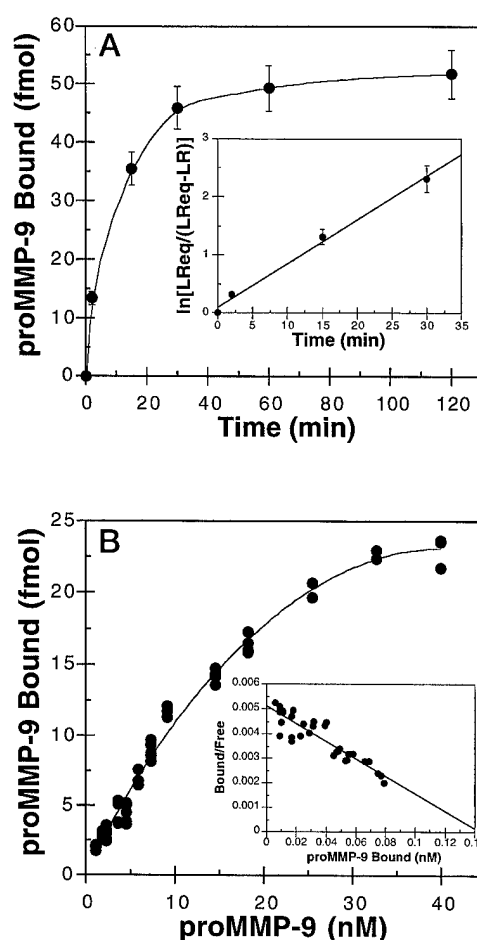


FIG. 1. **Binding of ^{125}I -pro-MMP-9.** A, time course of specific ^{125}I -pro-MMP-9 binding to MCF10A cells. Cells (2×10^5 cell/well) were incubated with 18 nM of ^{125}I -pro-MMP-9 for various times (0–120 min) at 4 °C in the presence or absence of 80-fold excess of unlabeled pro-MMP-9. At the indicated time points, the cells were processed to measure the quantity of cell-associated ^{125}I -pro-MMP-9 as described under "Experimental Procedures." The logarithmic transformation of the data is shown (inset) for calculation of the k_{on} . Each point represents triplicate wells with the standard deviation. B, dose dependence of specific ^{125}I -pro-MMP-9 binding to MCF10A cells. MCF10A cells were incubated (45 min at 4 °C) with increasing concentrations (1–40 nM) of ^{125}I -pro-MMP-9 in the presence or absence of 80-fold excess of unlabeled pro-MMP-9. At the end of the incubation period, the cells were lysed and the radioactivity measured to determine the quantity of cell-associated ^{125}I -pro-MMP-9. Every point, in triplicate, is presented. Inset in B, Scatchard plot analysis. This experiment was repeated at least three times with similar results.

chloroacetic acid-precipitable. Additionally, autoradiograms of the samples after SDS-PAGE showed no differences in cellular distribution between free and complexed zymogen (data not shown).

Identification of a 190-kDa Cell Surface Pro-MMP-9-binding Protein—The above binding data were consistent with the existence of a single pro-MMP-9-binding component on the cell surface. To identify the putative pro-MMP-9-binding protein, we carried out an affinity purification using immobilized pro-MMP-9 (Affi-Gel 10-pro-MMP-9). Lysates of surface-biotinylated MCF10A cells were incubated with Affi-Gel 10-pro-MMP-9 matrix or uncoupled Affi-Gel 10 and the bound proteins were eluted with 10% Me_2SO . The eluted proteins were then detected by either streptavidin-HRP or ligand blot analysis as described under "Experimental Procedures." Fig. 3 shows that MCF10A cells express a major 190-kDa protein that specifically and consistently binds to the Affi-Gel 10-pro-MMP-9 matrix (Fig. 3, A, lane 2; and B, lanes 2, 4, and 5) but not to the

uncoupled matrix (Fig. 3, A and B, lanes 1 and 3). Several minor biotinylated proteins (~70–90 kDa) were found to bind, albeit inconsistently, to the pro-MMP-9 affinity matrix (Fig. 3A, lane 2). The 190-kDa protein was detected by streptavidin-HRP (Fig. 3A, lane 2) consistent with biotinylation and consequently, cell surface localization. The streptavidin detection was specific since blots of samples derived from lysates of non-biotinylated cells and incubated with Affi-Gel 10-pro-

MMP-9 matrix were negative (Fig. 3A, lane 4). The presence of the 190-kDa protein in the non-biotinylated cells was confirmed by ligand blot analysis (Fig. 3B, lane 4). Electrophoretic migration of the 190-kDa protein was similar under nonreducing (Fig. 3B, lane 5) or reducing conditions (Fig. 3, A, lane 2, and B, lanes 2 and 4). In contrast, under nonreducing conditions, pro-MMP-9 exhibited the presence of monomer and dimer forms (Fig. 3B, lane 6), as expected (37).

To determine whether the 190-kDa protein forms a precipitable complex with pro-MMP-9, lysates of MCF10A cells were incubated with exogenous pro-MMP-9 and subjected to a co-immunoprecipitation experiment with anti-MMP-9 antibodies as described under "Experimental Procedures." The immunoprecipitates were analyzed by ligand blot analysis. This experiment showed that the 190-kDa protein co-precipitates with pro-MMP-9 (Fig. 3C). In the absence of enzyme, the 190-kDa protein was not detected, demonstrating the specificity of the interaction. Taken together, these results demonstrate that MCF10A cells express a 190-kDa cell surface protein that specifically binds to pro-MMP-9.

Affinity Purification of the 190-kDa Protein and Hydrodynamic Studies—The 190-kDa protein was purified from lysates of MCF10A cells using the Affi-Gel 10-pro-MMP-9 matrix as described under "Experimental Procedures." Fig. 4 shows the analysis of the column fractions in a silver-stained SDS-PAGE (Fig. 4A) and by ligand blot analysis (Fig. 4B). The 190-kDa protein was detected in the 20% Me₂SO eluate (Fig. 4, A and B, lane 5). Analysis of the protein content in each fraction indicated a ~3300-fold purification. The affinity-purified 190-kDa protein was subjected to hydrodynamic studies for determination of the sedimentation coefficient and Stokes radius to determine the native molecular mass. As shown in Fig. 5A, the 190-kDa protein was detected in six fractions derived from the gradient as shown by ligand blot analysis (Fig. 5A, inset). A sedimentation coefficient of 7.9 S was calculated from two determinations using known protein standards. The Stokes radius of the 190-kDa protein was determined by gel filtration using protein standards (Fig. 5B) and was calculated to be 52.6 Å. By combining the sedimentation and Stokes radius (38), a native molecular mass of 192,000 was calculated. These data and the electrophoretic mobility of the 190-kDa protein on SDS-PAGE (Fig. 3) indicate that this protein is monomeric.

The 190-kDa Pro-MMP-9-binding Protein Is the $\alpha 2(IV)$ Chain of Collagen IV—The affinity-purified 190-kDa protein was submitted for microsequencing as described under "Experimental Procedures." Analyses of three HPLC-purified peptides

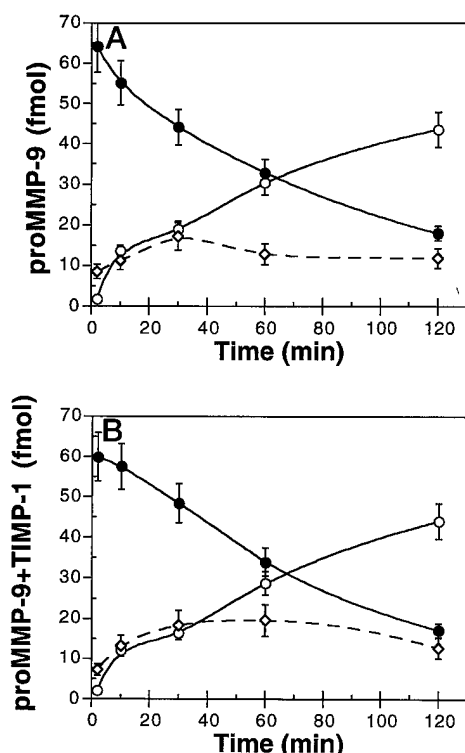


FIG. 2. Cellular distribution of ^{125}I -pro-MMP-9 and a ^{125}I -pro-MMP-9-TIMP-1 complex. Cells (2×10^5 cell/well) were incubated with either 18 nM of ^{125}I -pro-MMP-9 (A) or with a ^{125}I -pro-MMP-9-TIMP-1 complex (B) for 45 min at 4 °C. The cell monolayers were then washed with binding medium and incubated at 37 °C for various times (0–120 min). At each time point, the supernatant fraction, released enzyme (open circles), was collected and the cells were treated (30 min at 4 °C) with Pronase E, dislodged by gentle pipetting, and centrifuged to obtain the cell surface-bound ^{125}I -pro-MMP-9 (closed circles) and the internalized fraction (open diamonds). This experiment was performed four times with similar results.

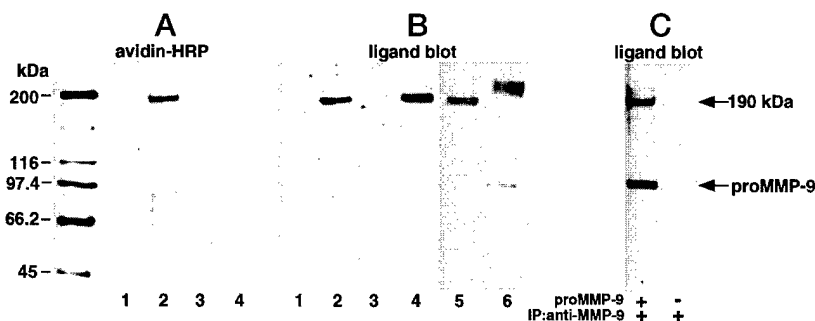


FIG. 3. Identification of a 190-kDa pro-MMP-9-binding protein. Lysates of surface-biotinylated (lanes 1 and 2) or non-biotinylated (lanes 3 and 4) MCF10A cells were incubated with either Affi-Gel 10-pro-MMP-9 matrix (lanes 2, 4, and 5) or uncoupled Affi-Gel 10 matrix (lanes 1 and 3) as described under "Experimental Procedures." The bound proteins were eluted with 10% Me₂SO in collagenase buffer, and the eluates were subjected to 4–12% SDS-PAGE under reducing (lanes 1–4) or nonreducing (lanes 5 and 6) conditions and transferred to a nitrocellulose membrane. Detection of the pro-MMP-9-binding protein was performed by either streptavidin-HRP (A) or ligand blot analysis (B) with pro-MMP-9 as a probe followed by detection with anti-MMP-9 antibody (CA-209) and anti-mouse IgG-HRP using ECL. Lane 6 shows the monomer and dimer forms of purified pro-MMP-9 (10 ng). C, co-precipitation of the 190-kDa protein with pro-MMP-9. A lysate (0.5 ml) of MCF10A cells was incubated (+) or not (–) with pro-MMP-9 (1 µg/ml), followed by addition of rabbit polyclonal anti-MMP-9 antibodies (5 µg) and then immunoprecipitated with protein G-Sepharose beads as described under "Experimental Procedures." The immunoprecipitates were subjected to 4–12% SDS-PAGE under reducing conditions, blotting to nitrocellulose and detection by ligand blot.

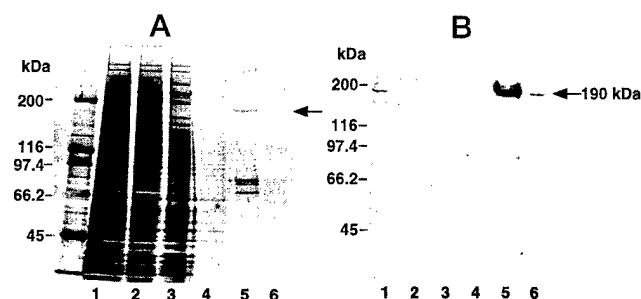


FIG. 4. Affinity purification of the 190-kDa protein. A lysate (132.5 mg) of MCF10A cells was subjected to affinity purification using Affi-Gel 10-pro-MMP-9 matrix as described under "Experimental Procedures." The collected fractions were subjected to 4–12% SDS-PAGE under reducing conditions and detection by either silver staining (A) or ligand blot analysis (B). Lane 1, crude lysate (26.5 μg); lane 2, flow-through (25 μg); lane 3, wash fraction 1 (17 μg); lane 4, wash fraction 2 (2.5 μg); lane 5, eluate fraction 1 (0.67 μg); lane 6, eluate fraction 2 (>0.05 μg).

obtained after tryptic digestion revealed the following amino acid sequences: GVSFGPGADGIPGHPGQGGP, DGYQGPDGPRG, and KIAIQPGTVGPQG, which correspond to residues 109–128, 325–335, and 1396–1408, respectively, of the human $\alpha 2(\text{IV})$ chain of collagen IV (39). Immunoblot analysis of the 190-kDa protein demonstrated reactivity with three different mAbs (H22, H25, and H21) against the human $\alpha 2(\text{IV})$ chain (Fig. 6A). Furthermore, co-immunoprecipitation of the 190-kDa protein from an MCF10A cell lysate with exogenous pro-MMP-9 using an anti-MMP-9 antibody followed by immunoblot analysis with H22 mAb further demonstrated that the coprecipitated 190-kDa protein is the $\alpha 2(\text{IV})$ chain (Fig. 6B).

Specificity of the Binding of ^{125}I -Pro-MMP-9 to $\alpha 2(\text{IV})$ —To evaluate the role of $\alpha 2(\text{IV})$ in the surface binding of pro-MMP-9, a binding assay was carried out with ^{125}I -pro-MMP-9 that was preincubated with affinity-purified $\alpha 2(\text{IV})$, as described under "Experimental Procedures." The results showed a 78% reduction in specific binding, from 4.5 to 1.0 fmol of pro-MMP-9 bound/ 1.3×10^5 cells, when ^{125}I -pro-MMP-9 (3.6 nM) was incubated with ~2-fold molar excess (~7 nM) of $\alpha 2(\text{IV})$ before the binding assay. Since $\alpha 2(\text{IV})$ is a substrate of MMP-9 (shown below), MCF10A cells were incubated (45 min, 37 °C) with MMP-9 (1.2 pmol/well) to remove surface-associated $\alpha 2(\text{IV})$. This treatment resulted in an 80% reduction in ^{125}I -pro-MMP-9-specific binding (from 6.4 to 1.4 fmol/ 1.3×10^5 cells). In contrast, when the cells were treated with MMP-9 in the presence of TIMP-1, a significant recovery of ^{125}I -pro-MMP-9-specific binding (from 1.4 to 4.4 fmol/ 1.3×10^5 cells) was observed, suggesting that TIMP-1 prevented the degradation of the surface-associated $\alpha 2(\text{IV})$. Collectively, these experiments suggest that the cell surface binding of pro-MMP-9 is mediated by $\alpha 2(\text{IV})$.

Expression of $\alpha 2(\text{IV})$ in Cultured Cells and Binding to Pro-MMP-9—We examined the expression of $\alpha 2(\text{IV})$ in cell extracts and on the surface of MCF10A, MDA-MB-231, HT1080, RVE, and CD3 cells by surface biotinylation, immunoblots and ligand blot analyses. Fig. 7 shows that all the cells examined express $\alpha 2(\text{IV})$ that specifically bound to the pro-MMP-9-affinity matrix (Fig. 7, A–D) or co-precipitated with pro-MMP-9 (Fig. 7E). Surface biotinylation followed by Affi-Gel 10-pro-MMP-9 purification demonstrated that $\alpha 2(\text{IV})$ is readily detected on the surface of MCF10A (Fig. 7B, lane 1), HT1080 (Fig. 7B, lane 3) and RVE (Fig. 7D) cells but could not be detected on the surface of MDA-MB-231 cells (Fig. 7B, lane 2). However, in the latter, $\alpha 2(\text{IV})$ was identified in the cell lysate (Fig. 7A, lane 2) consistent with the ligand binding data demonstrating a reduced number of pro-MMP-9

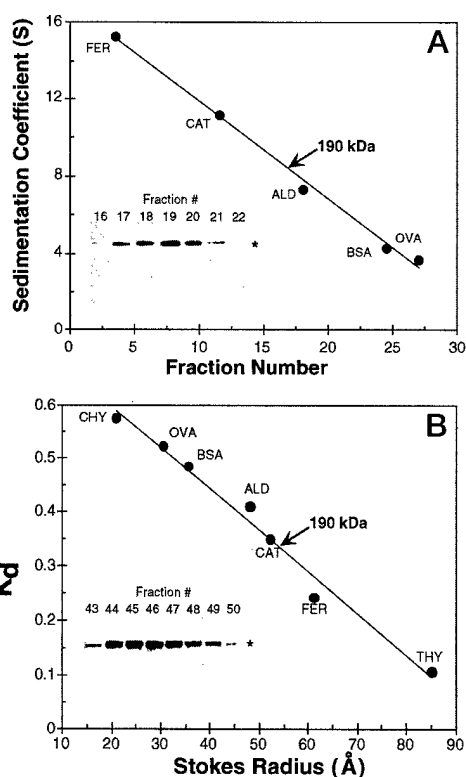


FIG. 5. Glycerol-gradient sedimentation and gel filtration of the 190-kDa protein. A, affinity-purified 190-kDa protein (500 ng) was sedimented in a 15–30% glycerol gradient as described under "Experimental Procedures." The 190-kDa-containing fractions were determined by ligand blot analysis (inset, asterisk). Protein markers run in parallel gradients were ferritin (FER, 15.3 S), catalase (CAT, 11.3 S), aldolase (ALD, 7.35 S), bovine serum albumin (BSA, 4.41 S), and ovalbumin (OVA, 3.7 S). B, the affinity-purified 190-kDa protein (250 ng) was chromatographed on a Superose 6 column as described under "Experimental Procedures." The 190-kDa-containing fractions were determined by SDS-PAGE under reducing conditions followed by ligand blot analysis (inset, asterisk). The protein markers indicated for Stokes radius determination were thyroglobulin (THY, 85 Å), ferritin (FER, 61 Å), catalase (CAT, 52.2 Å), aldolase (ALD, 48.1 Å), bovine serum albumin (BSA, 35.5 Å), ovalbumin (OVA, 30.5 Å), and chymotrypsin (CHY, 21 Å).

binding sites/cell in the MDA-MB-231 cells. Expression of $\alpha 2(\text{IV})$ was also detected in mouse endothelial CD3 cells by co-immunoprecipitation with pro-MMP-9 and detection by ligand blot analysis (Fig. 7E, panel 5). Thus, these studies demonstrate that monomeric $\alpha 2(\text{IV})$ is expressed by a variety of cells and can be detected on the cell surface.

Binding of Pro-MMP-9 to Collagen IV—The identification of $\alpha 2(\text{IV})$ and its binding to pro-MMP-9 raised the question about interactions of this enzyme with triple-helical collagen IV. Since the most abundant collagen IV is a heterotrimeric protein composed of two $\alpha 1(\text{IV})$ and one $\alpha 2(\text{IV})$ chains (40), we examined whether MCF10A cells produce trimeric collagen IV and whether it binds to pro-MMP-9. Concentrated serum-free conditioned medium of MCF10A cells was subjected to SDS-PAGE under nonreducing conditions, followed by immunoblot analysis using various antibodies to collagen IV. As shown in Fig. 8, mAbs to either the $\alpha 2(\text{IV})$ (Fig. 8, lane 1, mAb 1910) or $\alpha 1(\text{IV})$ (Fig. 8, lane 2, H11) chains recognized a protein of >450 kDa, likely to represent trimeric collagen IV. In contrast, H22 antibodies against $\alpha 2(\text{IV})$ did not react with trimeric collagen IV (Fig. 8, lane 3) suggesting that its epitope is not exposed under nonreducing conditions. A protein of ~160 kDa was also detected, although to a lower extent, with both H11 and H22 antibodies. The nature of this protein is unknown. It should be noted that, under these conditions, monomeric $\alpha 2(\text{IV})$ chain

could not be detected in the media of MCF10A cells by immunoblot and ligand blot analysis. Furthermore, growth of MCF10A cells in the presence of daily added ascorbate (75 μ g/ml) did not alter the localization and amounts of the $\alpha 2(IV)$ chain (data not shown).

The ability of pro-MMP-9 to bind collagen IV was tested by a co-immunoprecipitation experiment after the addition of pro-MMP-9 to the media of MCF10A cells. As control, pro-MMP-9 was also added to a cell lysate to co-precipitate $\alpha 2(IV)$. These studies demonstrated that, although the cell-associated $\alpha 2(IV)$ coprecipitated with pro-MMP-9 (Fig. 9A, lane 2), the secreted trimeric collagen IV did not (Fig. 9A, lane 1). Additionally, the collagen IV in the conditioned medium did not bind to the pro-MMP-9-affinity matrix (data not shown). The interaction of pro-MMP-9 with native collagen IV was further investigated using purified EHS collagen IV and the pro-MMP-9-affinity matrix as described under "Experimental Procedures." After the affinity step, the bound and unbound fractions were analyzed by ligand blot analysis under reducing conditions for the presence of mouse collagen IV chains. Fig. 9B shows that mouse collagen IV did not bind to the affinity matrix (Fig. 9B, lane 2). However, the $\alpha 1(IV)$ and $\alpha 2(IV)$ chains were readily detected in the unbound fraction (Fig. 9C, lane 2). It should be

noted that, under the conditions of the ligand blot analysis, the mouse $\alpha 1(IV)$ chain was detected. Taken together, these results suggest that pro-MMP-9 binds to monomeric $\alpha 2(IV)$ with an affinity greater than that to triple-helical collagen IV.

Domain Analysis of Pro-MMP-9- $\alpha 2(IV)$ Interactions—To define the pro-MMP-9 domains that interact with $\alpha 2(IV)$, we examined the binding of a pro-MMP-9-TIMP-1 complex since TIMP-1 is known to bind to the C-terminal domain of pro-MMP-9 (4, 37, 41). In addition, we questioned whether the active form of MMP-9, lacking the N-terminal domain, binds $\alpha 2(IV)$. A lysate of MCF10A cells was incubated with either a pro-MMP-9-TIMP-1 complex or with MMP-9. The proteins were then immunoprecipitated with either anti-MMP-9 or anti-TIMP-1 antibodies and the immunoprecipitates were resolved by ligand blot analysis. As shown in Fig. 10, $\alpha 2(IV)$ coprecipitated with pro-MMP-9, as expected (Fig. 10A, lane 1). In contrast, TIMP-1 alone did not bind to $\alpha 2(IV)$ (Fig. 10A, lane 2). In the presence of a pro-MMP-9-TIMP-1 complex, $\alpha 2(IV)$ was co-immunoprecipitated by either anti-TIMP-1 (Fig. 10A, lane 4) or anti-MMP-9 antibodies (Fig. 10A, lane 3). Thus, the pro-MMP-9-TIMP-1 complex interacts with $\alpha 2(IV)$. Development of the same blot with anti-TIMP-1 antibodies (Fig. 10B, lanes 5–8) showed TIMP-1 in the samples containing inhibitor (Fig. 10B, lanes 6–8). When MMP-9 was added to MCF10A lysates, $\alpha 2(IV)$ could not be detected in the immunoprecipitate (Fig. 10C, lane 9) or in the supernatant fraction of the immunoprecipitates suggesting that it was probably degraded by MMP-9. However, $\alpha 2(IV)$ coprecipitated with the active enzyme (Fig. 10C, lane 10) when the experiment was carried out in the presence of 10 mM EDTA. Thus, neither the N-terminal nor the C-terminal domain (at least the region of TIMP-1 interaction) of pro-MMP-9 appears to be critical for binding to $\alpha 2(IV)$. However, we have consistently found that a pro-MMP-9- $\alpha 2(IV)$ complex failed to co-precipitate with gelatin-agarose matrix (data not shown), suggesting a role for the gelatin-binding domain in the interaction of pro-MMP-9 with $\alpha 2(IV)$.

Affinity of Pro-MMP-9 and Pro-MMP-2 for $\alpha 2(IV)$ —Since pro-MMP-9 bears a high degree of sequence similarity with pro-MMP-2 and both possess a gelatin-binding domain (41), we determined the ability of pro-MMP-2 to compete for the binding of $\alpha 2(IV)$ to pro-MMP-9. The $\alpha 2(IV)$ was co-immunoprecipitated with pro-MMP-9 and anti-MMP-9 antibodies in the absence or presence of pro-MMP-2 (equimolar or 5-fold molar excess, relative to pro-MMP-9) and in the presence of EDTA to prevent degradation of $\alpha 2(IV)$ by any trace of MMP-2. The immunoprecipitates were resolved by ligand blot analysis. As shown in Fig. 11, $\alpha 2(IV)$ co-precipitates only in the presence of pro-MMP-9

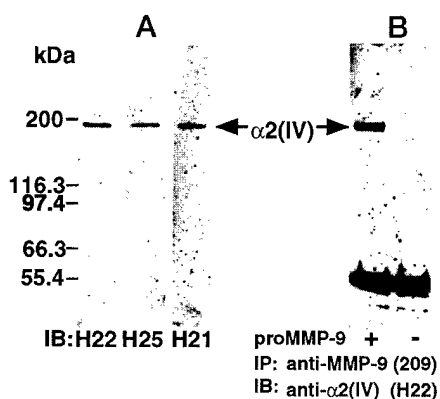


FIG. 6. Immunoblot analysis of the 190-kDa protein with anti- $\alpha 2(IV)$ antibodies. A, affinity-purified $\alpha 2(IV)$ (50 ng/lane) was subjected to 4–12% SDS-PAGE under reducing conditions followed by immunoblot analysis with three different mAbs, H22, H25 and H21, to the human $\alpha 2(IV)$ chain and detection by ECL. B, a lysate (1 ml) of MCF10A cells prepared as described under "Experimental Procedures" was incubated (1 h, 4 °C) with (+) or without (-) pro-MMP-9 (0.5 μ g/ml) and then immunoprecipitated with anti-MMP-9 antibodies and protein G-Sepharose beads. The immunoprecipitates were resolved by 4–12% SDS-PAGE under reducing conditions, followed by immunoblot analysis with H22 antibodies and detection by ECL. IP, immunoprecipitation; IB, immunoblot.

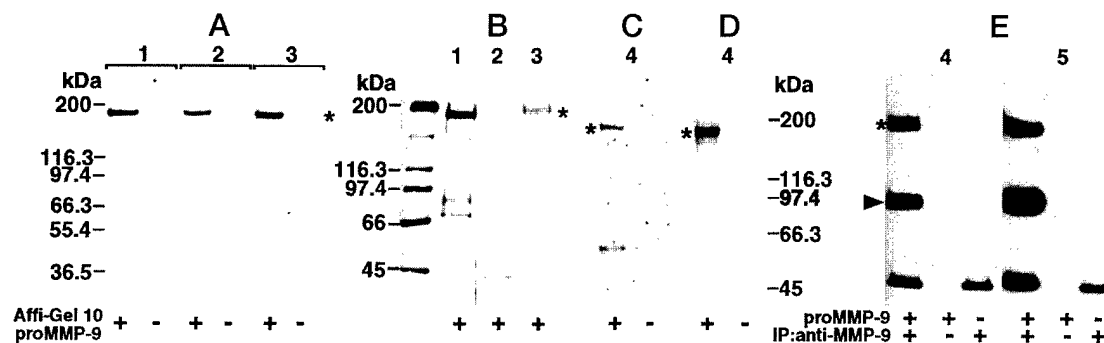


FIG. 7. Expression of $\alpha 2(IV)$ in cultured cells. MCF10A (lane 1), MDA-MB-231 (lane 2), HT1080 (lane 3), RVE (panel 4), and CD3 (panel 5) cells were examined for $\alpha 2(IV)$ expression in lysates of non-biotinylated (A, C, and E) or surface-biotinylated cells (B and D). Samples were incubated with either Affi-Gel 10-pro-MMP-9 (+) or Affi-Gel 10 alone (-) (A–D) or subjected to a co-immunoprecipitation with pro-MMP-9 (+) and anti-pro-MMP-9 antibodies (+) (E). Detection of $\alpha 2(IV)$ was achieved by immunoblot with anti- $\alpha 2(IV)$ antibodies (A and C) or streptavidin-HRP (B and D), or by ligand blot analyses with pro-MMP-9 as a probe (E). Asterisks indicate the position of $\alpha 2(IV)$. Arrow shows pro-MMP-9 detected in the ligand blot.

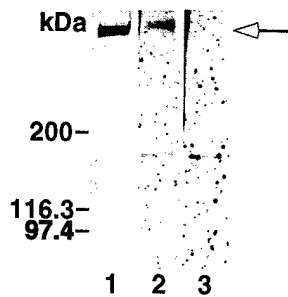


FIG. 8. Expression of collagen IV in the media of MCF10A cells. Aliquots (20 μl) of concentrated serum-free conditioned media of MCF10A cells were subjected to 4–12% SDS-PAGE under nonreducing conditions followed by transfer to a nitrocellulose membrane. Collagen IV was probed with mAbs against either $\alpha 2(\text{IV})$ (mAb 1910 (lane 1) and H22 (lane 3)) or $\alpha 1(\text{IV})$ (H11 (lane 2)), and detection was performed using ECL as described under "Experimental Procedures." The arrow demonstrates the position of the trimeric collagen IV of >450 kDa.

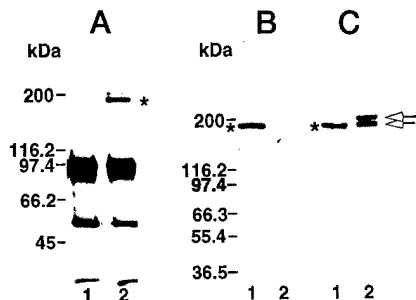


FIG. 9. Binding of pro-MMP-9 to collagen IV. A, concentrated serum-free conditioned medium (lane 1) or a lysate (lane 2) of MCF10A cells were incubated (1 h, 4 °C) with pro-MMP-9 (0.5 $\mu\text{g}/\text{ml}$) and then immunoprecipitated with anti-MMP-9 antibodies and protein G-Sepharose beads. The immunoprecipitates were resolved by 4–12% SDS-PAGE under reducing conditions followed by ligand blot analysis. B and C, EHS collagen IV (10 $\mu\text{g}/\text{ml}$) was incubated (overnight, 4 °C) with the Affi-Gel 10-pro-MMP-9 matrix (30 μl) in lysis buffer (0.5 ml, final volume). After incubation, the supernatant was collected and the beads were processed as described under "Experimental Procedures." The proteins in the bound (B) and the unbound (C) fractions were resolved by 4–12% SDS-PAGE under reducing conditions followed by ligand blot analysis. B, lane 2, bound fraction; C, lane 2, unbound fraction. Asterisks show the $\alpha 2(\text{IV})$ chain. Open arrows in C indicate the position of the $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains of mouse EHS collagen IV. Lane 1 in B and C shows the affinity-purified $\alpha 2(\text{IV})$, as a control.

(Fig. 11A, lanes 2–4). No significant differences in the amounts of $\alpha 2(\text{IV})$ co-precipitating with pro-MMP-9 were detected in the presence of equal molar amounts (Fig. 11A, lane 3) or excess (5-fold) molar amounts of pro-MMP-2 (Fig. 11A, lane 4), suggesting that under these conditions only pro-MMP-9 bound to $\alpha 2(\text{IV})$. In agreement with these results, $\alpha 2(\text{IV})$ incubated with pro-MMP-2 alone did not form a co-precipitable complex as determined using anti-MMP-2 antibodies and ligand blot analysis (Fig. 11A, lane 6). Consistently, pro-MMP-2 was detected in the immunoprecipitates when the same blot was developed with anti-MMP-2 antibodies (Fig. 11B, lane 8).

To obtain a quantitative measurement of the relative affinities of pro-MMP-9 and pro-MMP-2 for the $\alpha 2(\text{IV})$ chain, we examined the ability of $\alpha 2(\text{IV})$ to form a complex with either pro-MMP-2 or pro-MMP-9 by co-chromatography and the dissociation constants (K_d) were determined as described under "Experimental Procedures." Fig. 12 shows that both pro-MMP-9 and pro-MMP-2 bind $\alpha 2(\text{IV})$ as a function of proenzyme concentration. Under these conditions, maximal binding was observed at concentrations of enzymes of ~ 150 nM for pro-MMP-9 and >750 nM for pro-MMP-2. From these data, the K_d values of $\alpha 2(\text{IV})$ for pro-MMP-9 and pro-MMP-2

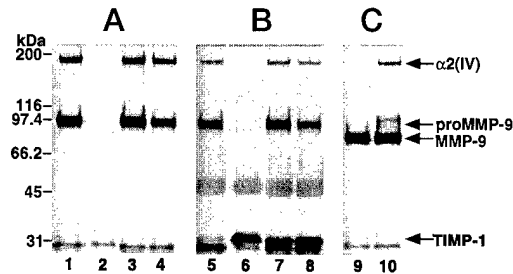


FIG. 10. Binding of a pro-MMP-9-TIMP-1 complex and MMP-9 to $\alpha 2(\text{IV})$. Lysates (0.5 ml) of MCF10A cells were incubated (1 h, 4 °C) with either pro-MMP-9 (0.5 $\mu\text{g}/\text{ml}$) (lanes 1 and 5), TIMP-1 (0.33 $\mu\text{g}/\text{ml}$) (lanes 2 and 6), a pro-MMP-9-TIMP-1 complex (lanes 3, 4, 7, and 8) or with MMP-9 (0.5 $\mu\text{g}/\text{ml}$) (lanes 9 and 10) in the absence (lanes 1–9) or presence (lane 10) of 5 mM EDTA. The samples were immunoprecipitated with mAbs to either pro-MMP-9 (CA-209) (lanes 1, 3, 5, 7, 9, and 10) or TIMP-1 (IM 322) (lanes 2, 4, 6, and 8). The immunoprecipitates were resolved by 4–12% SDS-PAGE under reducing conditions and the proteins detected by ligand blot analysis (A and C). The membrane in A was subsequently developed by immunoblot analysis (B) using a rabbit anti-TIMP-1 polyclonal antibody. The proteins of approximately 55 and 20 kDa are the heavy and light chains of the IgG molecule, respectively.

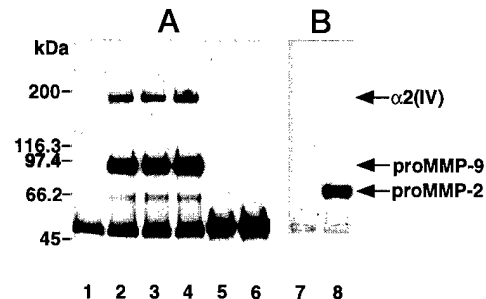


FIG. 11. Co-immunoprecipitation of pro-MMP-9 with $\alpha 2(\text{IV})$ and competition with pro-MMP-2. Affinity-purified $\alpha 2(\text{IV})$ (~ 0.5 μg) was incubated (1 h, 4 °C) without (lanes 1, 5, and 7) or with 5 nM pro-MMP-9 (lanes 2–4) or with 5 nM pro-MMP-2 (lanes 6 and 8) in the presence of 10 mM EDTA. The samples in lanes 3 and 4 also received 5 nM (1:1 pro-MMP-9:pro-MMP-2 molar ratio) and 25 nM pro-MMP-2 (1:5 pro-MMP-9:pro-MMP-2 molar ratio), respectively. The samples were immunoprecipitated with either anti-pro-MMP-9 (CA-209) (lanes 1–4) or anti-MMP-2 (CA-801 and CA-805) (lanes 5–8) antibodies as described under "Experimental Procedures." A, the immunoprecipitates were resolved by 4–12% SDS-PAGE under reducing conditions followed by ligand blot analysis. B, the samples in lanes 5 and 6 from A were examined by immunoblot analysis using a polyclonal antibody to pro-MMP-2. The protein of approximately 55 kDa is the heavy chain of the IgG molecule.

were calculated to be approximately 45 nM and ≥ 350 nM, respectively.

DISCUSSION

Previously, we have shown the surface association of pro-MMP-9 in 12-*O*-tetradecanoylphorbol-13-acetate-treated MCF10A cells (25). Other studies have described the presence of pro-MMP-9 on plasma membranes of HT1080 cells (23) and in focal contacts of endothelial cells (22). Here, we have characterized the binding parameters of pro-MMP-9 and demonstrated that the proenzyme binds with high affinity ($K_d \sim 20$ –30 nM) to the surface of a variety of cell types. In MCF10A cells, internalization studies demonstrated that binding of pro-MMP-9, free or in complex with TIMP-1, is not followed by ligand internalization. Furthermore, under the experimental conditions, surface binding of pro-MMP-9 does not result in zymogen activation, possibly due to the lack of activators in the cell culture system. However, recent studies demonstrated that surface-associated pro-MMP-9 can be activated by a plasmin-dependent mechanism (23, 24). Taken together, these findings

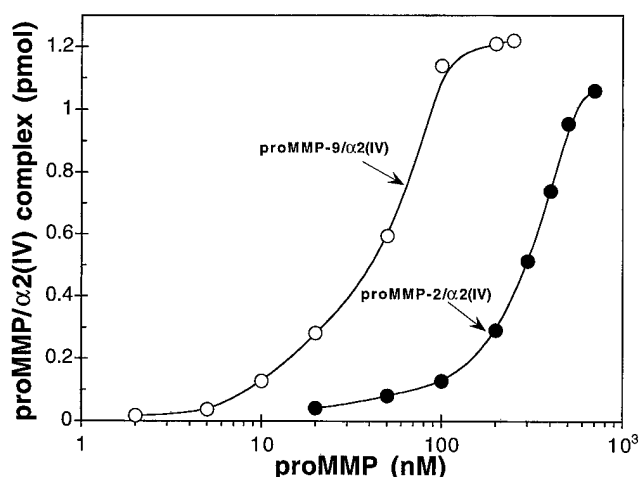


FIG. 12. Gel filtration of pro-MMP-9- $\alpha 2(\text{IV})$ and pro-MMP-2- $\alpha 2(\text{IV})$ complexes. Affinity-purified $\alpha 2(\text{IV})$ (9.5 nM) was allowed to complex with increasing concentrations of either ^{35}S -pro-MMP-9 (2.5 to 250 nM) or ^{35}S -pro-MMP-2 (20 to 750 nM) in a final volume of 100 μl as described under "Experimental Procedures." The samples were subjected to Superose 6 gel filtration chromatography, and the amount of pro-MMP-9 and pro-MMP-2 complexed with $\alpha 2(\text{IV})$ was determined.

suggest that the surface association of pro-MMP-9 is not directly associated with activation and/or internalization but plays a role in confinement of proenzyme in areas of cell-matrix contacts, where it could become the target for potential pro-MMP-9-activating proteinases.

We identified a 190-kDa cell surface protein in various cell lines that specifically bound to a pro-MMP-9-affinity matrix and formed a co-precipitable complex with pro-MMP-9. Microsequencing of the affinity-purified protein revealed a complete sequence homology to the $\alpha 2(\text{IV})$ chain of human collagen IV (39). Additionally, immunoblot analysis with chain-specific antibodies (30) corroborated that the 190-kDa protein is $\alpha 2(\text{IV})$. Several observations indicate that the cell surface association of pro-MMP-9 is mediated by $\alpha 2(\text{IV})$. (i) Preincubation of pro-MMP-9 with affinity-purified $\alpha 2(\text{IV})$ significantly inhibited ligand binding. (ii) Pretreatment of MCF10A cells with MMP-9 reduced by 80% the binding of proenzyme, likely due to the degradation of surface-associated $\alpha 2(\text{IV})$ as observed in experiments with soluble $\alpha 2(\text{IV})$ and MMP-9. Accordingly, treatment of cells with MMP-9 and TIMP-1 resulted in the recovery of the majority of ligand binding; (iii) Binding of pro-MMP-9 to the cells exhibited an affinity ($K_d \sim 22$ nM) that was in close agreement with the affinity ($K_d \sim 45$ nM) of the enzyme for purified $\alpha 2(\text{IV})$. In addition, there was a good correlation between the number of pro-MMP-9 binding sites determined in the binding assays and the level of expression of surface-associated $\alpha 2(\text{IV})$. For example, the breast cancer MDA-MB-231 cells that exhibited 10-fold less binding sites than MCF10A cells showed undetectable levels of surface-associated $\alpha 2(\text{IV})$. The reason for the low levels of $\alpha 2(\text{IV})$ on the surface of these malignant cells is unknown but may be related to their inability to retain $\alpha 2(\text{IV})$ on the cell surface since $\alpha 2(\text{IV})$ was detected intracellularly. Thus, *in vitro*, the surface association of pro-MMP-9 would depend on the ability of each cell type to express and retain $\alpha 2(\text{IV})$ on the cell surface. Whether pro-MMP-9 and pro-MMP-2 bind also to monomeric $\alpha 1(\text{IV})$ is yet unknown and remains to be determined. However, the fact that only a single polypeptide, $\alpha 2(\text{IV})$, was specifically and consistently co-precipitated with pro-MMP-9 or bound to immobilized proenzyme suggests a unique and preferential interaction between pro-MMP-9 and $\alpha 2(\text{IV})$.

Co-immunoprecipitation experiments using a pro-MMP-

9-TIMP-1 complex or MMP-9 demonstrated that neither the C-terminal nor the N-terminal domains of pro-MMP-9 appeared to be critical for interactions with $\alpha 2(\text{IV})$. However, we could not precipitate the $\alpha 2(\text{IV})$ /pro-MMP-9 complex with gelatin-agarose beads, suggesting a role for the gelatin-binding domain. In this regard, it was interesting to observe that pro-MMP-2, when compared with pro-MMP-9, exhibited a lower affinity for $\alpha 2(\text{IV})$ as determined by the co-immunoprecipitation and gel filtration experiments. Since pro-MMP-2 contains a similar gelatin binding domain (41), this suggests that the interactions of $\alpha 2(\text{IV})$ with pro-MMP-9 are also influenced by sites/domains other than the gelatin binding domain and/or by the three-dimensional conformation of pro-MMP-9. It would be of interest to determine whether the 54-amino acid proline-rich $\alpha 2(\text{V})$ -like extension that is uniquely present in pro-MMP-9 (17, 41), plays any role in the binding of the enzyme to $\alpha 2(\text{IV})$. In the cell binding assays, pro-MMP-2 slightly competed with pro-MMP-9 binding, suggesting that surface-associated $\alpha 2(\text{IV})$ is also available for pro-MMP-2 binding. However, given the lower affinity of pro-MMP-2 for $\alpha 2(\text{IV})$ and the existence of alternate high affinity surface binding sites for pro-MMP-2 (14), this enzyme would be expected to bind to the cell surface via a different mechanism (14, 16).

The binding of gelatinases to collagen IV has been examined in previous studies (21, 42); however, the sites of interaction were not defined. The results presented here suggest that a major high affinity binding site for pro-MMP-9 in collagen IV must reside within the $\alpha 2(\text{IV})$ chain. However, our data also indicate that neither triple-helical collagen IV secreted by MCF10A cells nor EHS collagen IV bound to pro-MMP-9 under conditions of $\alpha 2(\text{IV})$ binding. In agreement with these results, we have recently observed a weak affinity ($K_d = 2.15 \mu\text{M}$) of pro-MMP-9 for EHS collagen IV as determined by surface plasmon resonance⁴. Thus, whereas monomeric $\alpha 2(\text{IV})$ forms a tight complex with pro-MMP-9 (K_d nM), trimeric collagen IV binds pro-MMP-9 with very low affinity ($K_d \mu\text{M}$). This is also consistent with the studies of Steffensen *et al.* (21), who showed a weaker affinity of a recombinant gelatin-binding domain of pro-MMP-2 for trimeric collagen IV compared with that for denatured collagen IV. This has led to the suggestion that binding sites for the gelatin-binding domain in denatured collagen IV may be masked in triple-helical collagen IV (21). Therefore, it is conceivable that the binding of pro-MMP-9 to $\alpha 2(\text{IV})$ is mediated by sites that are cryptic in collagen IV and that are exposed after partial denaturation and/or degradation of the collagen IV molecule. If so, these sites would have to be conserved after partial proteolysis to allow binding of pro-MMP-9. Such a scenario would probably involve a partial degradation of the collagen IV network by a protease(s) other than the gelatinases as the catalytic efficiency of MMP-2 and MMP-9 against native collagen IV has been reported to be limited (43–45). After collagen IV degradation, secretion of pro-MMP-9 by pro-MMP-9 producing-cells in close association with the basement membrane would then facilitate binding of pro-MMP-9 to $\alpha 2(\text{IV})$. After activation of the $\alpha 2(\text{IV})$ -bound pro-MMP-9, the enzyme would then contribute to the complete degradation of the collagen IV network consistent with its ability to degrade denatured collagens (41).

Another possible scenario for the results observed here may involve binding of pro-MMP-9 to a surface-associated $\alpha 2(\text{IV})$. Although the origin and binding mechanism of $\alpha 2(\text{IV})$ chains to the cell surface remain to be elucidated, our *in vitro* studies with a variety of cell lines (epithelial, endothelial and fibrosarcoma) clearly show that, although some $\alpha 2(\text{IV})$ chains are as-

⁴ M. Olson and R. Fridman, unpublished results.

sembled with $\alpha 1(\text{IV})$ into trimeric collagen IV molecules, a portion of single $\alpha 2(\text{IV})$ chains are secreted and deposited on the cell surface. The surface-associated $\alpha 2(\text{IV})$ chains appear to be stable as determined by surface biotinylation and pulse-chase analysis. Indeed, we have found that $\alpha 2(\text{IV})$ chains can be detected in the cellular compartment of both MCF10A and HT1080 cells and in the supernatant of the latter even after a 10-h chase period without any evidence of processing and/or degradation.⁵ These findings are in contrast with the general notion that $\alpha(\text{IV})$ chains that fail to form trimeric collagen molecules are unstable and/or rapidly hydrolyzed. This is generally true if active proteinases are present in the experimental system, as they can hydrolyze non-triple-helical collagen chains as shown here with $\alpha 2(\text{IV})$ and MMP-9. Thus, the integrity and eventually the detection of $\alpha(\text{IV})$ chains would depend on the presence of active proteinases and/or inhibitors in each particular culture system and tissue. Detection of monomeric $\alpha(\text{IV})$ chains may also depend on the extraction procedures and/or on the antibodies used since some antibodies may only recognize $\alpha(\text{IV})$ chains in triple-helical conformation. Here we have used immobilized pro-MMP-9 and chain-specific mAbs raised against synthetic peptides, which independently allowed for the detection of native and stable $\alpha 2(\text{IV})$ chains on the cell surface. Although the processing, localization and stability of monomeric $\alpha 2(\text{IV})$ warrants further *in vitro* and *in vivo* studies, it is tempting to speculate that surface-associated $\alpha 2(\text{IV})$ may anchor pro-MMP-9 on the cell surface after autocrine or paracrine secretion. After activation by pro-MMP-9-activating enzymes, MMP-9 would then play a role in localized surface proteolysis. Since the binding of pro-MMP-9 to the $\alpha 2(\text{IV})$ chain is of high affinity and involves the zymogen form, it will be important in future studies to address the activation of pro-MMP-9 and its interactions with TIMP-1 in the context of pro-MMP-9 bound to $\alpha 2(\text{IV})$.

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⁵ M. Toth and R. Fridman, unpublished results.

16. Cathepsins D and B in breast cancer

Wei-Ping Ren and Bonnie F. Sloane

Introduction

The name *cathepsin* is derived from a Greek word meaning to digest and was used originally for acidic proteases, with the letters designating individual enzymes [1,2]. Examples of cathepsins are found in three classes of proteases, for example, cathepsin D is an aspartic protease, cathepsin B a cysteine protease, and cathepsin G is a serine protease. Although several cathepsins have collagenolytic activity, there are no cathepsins among the metalloproteases. Most cathepsins are lysosomal proteases with acidic pH optima ranging from the extremely acidic pH optimum of cathepsin D (i.e., pH 2.8) to the slightly acidic pH optimum of cathepsin B (i.e., pH 6.5). On the other hand, cathepsin E has an acidic pH optimum (i.e., pH 3.0) yet is not lysosomal [3], and cathepsin S has a neutral pH optimum yet is lysosomal [4]. The lysosomal cathepsins are synthesized as pre-proenzymes and acquire N-linked oligosaccharides cotranslationally. Their maturation involves proteolytic processing and modification of the oligosaccharides, processes that occur during their trafficking to the lysosome and that affect their ability to bind to receptors that target them to the lysosomes [5-7]. As indicated earlier, the cathepsins are primarily endopeptidases, hydrolyzing internal peptide bonds. However, cathepsin H is an exopeptidase of the aminopeptidase class [8], and cathepsin B has both endopeptidase and exopeptidase activities, the latter as a carboxydipeptidase [9]. The dual activity of cathepsin B enables this enzyme to participate in degradatory processes directly through degradation of the basement membrane proteins laminin, fibronectin, and type IV collagen [10,11], or indirectly by activating other proteases such as urokinase [12]. The primary intracellular function of lysosomal cathepsins is protein turnover. The evidence for extracellular roles of cathepsins is growing; as an example, cathepsins secreted from macrophages and osteoclasts participate in matrix and bone degradation [13,14].

Cathepsins have also been implicated in several processes, presumably extracellular, that accompany tumor progression: proliferation, angiogenesis, invasion, and metastasis [2]. Surprisingly, the association of cathepsins D and B with malignancy of human breast tumors was first described almost 15 years

Table 1. General information on cathepsin D and cathepsin B

	Cathepsin D	Cathepsin B
Molecular size		
Proform	43 kD ^a (52 kD ^b)	35 kD (43/46 kD)
Mature single chain	38 kD (48 kD)	28 kD (31 kD)
Mature double chain	26 + 12 kD (34 + 14 kD)	22 + 6 kD (25/26 + 5 kD)
Assay substrate	Denatured hemoglobin	Carbobenzyloxy-arginyl-arginyl-4-methylcoumarin
pH optimum	pH 3.0	pH 6.5
Inhibitors		
Endogenous	None	Cystatin superfamily
Exogenous	Pepstatin	E-64, leupeptin, CA074 and CA074Me ^c

^aMolecular sizes are calculated from amino acid sequences predicted from the nucleotide sequences of human cathepsin D [22] and cathepsin B [49], using an average molecular weight of 110 daltons per amino acid.

^bMolecular sizes in parentheses represent sizes of glycosylated enzymes immunoprecipitated from metabolically labeled cells [141,142].

^cE-64 and leupeptin will inhibit cathepsin B, as well as other cysteine proteases, whereas CA074 and CA074Me are selective for cathepsin B.

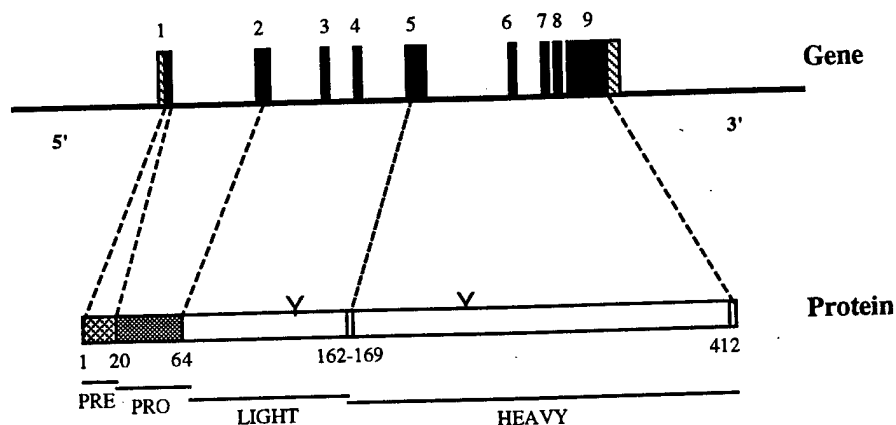
ago [15–17]. As reviews of earlier literature linking these enzymes to the progression of human cancers are available [2,18–21], we will concentrate in this chapter on the most recent data, with an emphasis on the data linking these enzymes to the progression of human breast cancer. To provide a framework for readers unfamiliar with these two enzymes, in Figure 1 we have diagrammed the gene and protein structures of cathepsins D and B, and in Table 1 we have provided information on the characteristics of both enzymes.

Increased expression of cathepsins D and B

Cathepsin D

The cathepsin D gene is a single copy gene comprised of nine exons and eight introns spanning a region of 11 kb [22,23] (Figure 1). It is located on the short arm of chromosome 11 close to the *Ha-ras* oncogene. This is a region that undergoes frequent rearrangements, including the loss of one *c-Ha-ras* allele, in aggressive breast cancer [24]. The translation initiation site of cathepsin D is encoded by exon 1 and the stop codon by exon 9 [22]. The deduced amino acid sequence [22] indicates that human cathepsin D consists of a pre-proenzyme with a 20-residue signal peptide, a 44-residue propeptide, and a single chain form of 348 residues. The human cathepsin D gene has a compound promoter with features of both housekeeping (high G+C content and potential Sp-1 transcription factor binding sites) and regulated (the presence of a TATA box) promoters [25]. Five transcription start sites (TSSI to V)

Cathepsin D



Cathepsin B

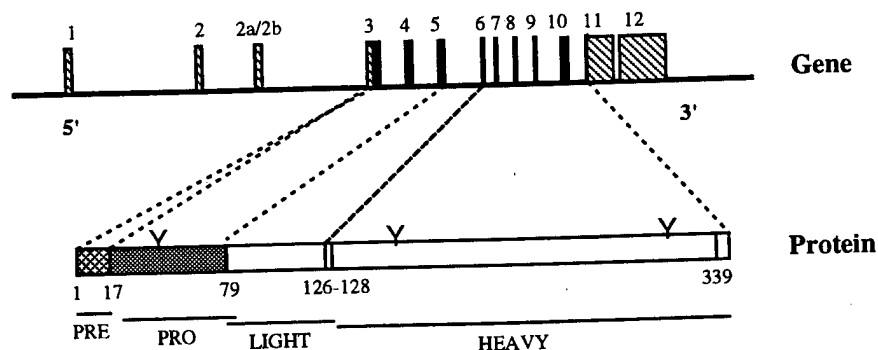


Figure 1. Comparison of the gene and protein structures of cathepsin D and cathepsin B. The human cathepsin D structure is based on Redecker et al. [22], and the human cathepsin B structure is based on Gong et al. [47] and Berquin et al. [48]. Exons are numbered; those encoding the protein are depicted by filled boxes, whereas those representing 5'- and 3'-untranslated regions are depicted by striped boxes. In the protein structures, vertical lines indicate sites of proteolytic processing and Ys indicate putative glycosylation sites. The number of amino acids in the prepeptide, propeptide, and light and heavy chains of mature cathepsin D and cathepsin B are indicated underneath the protein structure.

spanning >52bp have been identified by RNase protection assay [25]. In estrogen-dependent breast cancer cells, estradiol induces a 6- to 10-fold increase in cathepsin D transcripts; these transcripts are initiated at TSSI located about 28bp downstream from the TATA box. Site-directed mutagenesis indi-

cates that the TATA box is essential for initiation of transcription at TSSI [25]. In breast cancer biopsies, high levels of TATA-dependent transcription correlate with overexpression of cathepsin D transcripts (see later). Thus, the cathepsin D gene can behave as either a housekeeping gene with multiple start sites or as a hormone-regulated gene with a single TATA-dependent start site [25].

The major mRNA transcript for cathepsin D, observed in both normal and breast tumor cells, is 2.2 kb [22,23,26]. Estrogen regulates the expression of the cathepsin D gene primarily at the transcriptional level [26-28], as has been observed for other steroid-responsive genes. At least two estrogen-responsive elements are located upstream of TSSI in the cathepsin D gene [29]. In MCF-7 human breast cancer cells, an estrogen receptor-positive (ER⁺) cell line, there is a low constitutive accumulation of cathepsin D mRNA, which is increased about 10-fold by estradiol treatment [26]. The estradiol-induced cathepsin D expression is not inhibited by cycloheximide, suggesting that regulation by estrogen does not involve other effector proteins [26]. Estrogen receptor ligands are the only steroids that induce transcription; the ability of the estrogen receptor ligands to induce transcription parallels their affinity for the estrogen receptor and their mitogenic activity. The promoter has not yet been compared in normal and cancer tissues. Therefore, whether the increased sensitivity to estradiol in breast cancer is due to altered *trans*-acting factors or altered *cis*-acting sequences has not been determined.

The expression of cathepsin D is also increased in estrogen receptor-negative (ER⁻) breast cancers [27]. The mechanism is still unknown, but the constitutive production of autocrine or intracrine factor(s) might be responsible for the induction of cathepsin D. Although Rochefort and coworkers [26-28] have shown that growth factors, such as insulin-like growth factor 1, epidermal growth factor, and basic fibroblast growth factor, acting via a tyrosine kinase pathway, are able to induce cathepsin D mRNA in the ER⁺ MCF-7 cells, the role(s) of growth factors in constitutive overexpression of cathepsin D in ER⁻ breast cancers has not been delineated. The induction of cathepsin D expression by growth factors, in contrast to the induction by estrogen, is inhibited by cycloheximide [27], indicating an indirect mechanism of action.

Increases in the expression of cathepsin D have been observed in breast cancer tissues and cell lines at both the mRNA [26,27,30] and protein [31] levels. Immunohistochemistry [31,32], in situ hybridization and cytosolic immunoassays [33], and northern and western blot analyses [28,34] all indicate that cathepsin D expression is 2- to 50-fold greater in most cases of breast cancer than in normal mammary glands or in fibroblasts. In situ hybridization and immunohistochemistry have established that the increases in expression occur in the breast cancer cells rather than in stromal cells [35-37]; expression in stromal cells has also been reported for urokinase [38] and some metalloproteases [39]. Although macrophages also express cathepsin D at high levels [40], the expression of cathepsin D in macrophages within the

breast cancer tissue does not seem to account for the 2- to 50-fold higher expression of cathepsin D [41]. Increases in expression of cathepsin D are not unique to breast cancer; such increases have also been observed in hepatomas [42], brain tumors [43], and thyroid carcinomas [44].

Cathepsin B

The human cathepsin B gene is a single copy gene that maps to chromosome 8p22 [45]. This is a region that is a hot spot in prostate cancer [46]. The human cathepsin B gene is composed of at least 12 exons [47,48], spanning a region of approximately 27 kb [48] (Figure 1). The gene structure of cathepsin B does not correspond to the functional units of the enzyme [2]. The translation initiation site is in exon 3, so that exon 1, 2, and 25 bp of exon 3 are noncoding and constitute the 5'-untranslated region (UTR). Exon 12 and about 80% of exon 11 are also noncoding and make up the 3'-UTR. Alternative splicing of both the 5'- and 3'-UTRs has been observed [47-50]. We recently identified two new exons in the human cathepsin B gene that map between exons 2 and 3 [48]. These two exons can be alternatively spliced and contribute to the diversity of the 5'-UTR of cathepsin B transcripts. The deduced amino acid sequence indicates that cathepsin B transcripts encode a pre-proenzyme with a 17-residue signal peptide, a 62-residue propeptide, and a 254 residue single chain mature form [51] (Figure 1). The single chain form of cathepsin B is processed to the double-chain form by two internal cleavages, which eliminate a dipeptide. Both single and double chain forms of the enzyme have proteolytic activity [52].

The putative promoter region ≤ 500 bp upstream of exon 1 of the human cathepsin B gene does not have TATA or CAAT motifs, but has a high G+C content and several potential binding sites for the Sp-1 transcription factor [47]. Therefore, the promoter of the human cathepsin B gene has been classified as a housekeeping-type promoter, as has that for the mouse cathepsin B gene [53]. More recent studies suggest that both the mouse [54] and human [48] cathepsin B genes may have more than one promoter. In the mouse, two newly identified putative promoter regions contain a TATA box, with one also containing a CAAT box. The sequence of the two putative promoter regions in the human cathepsin B gene is not yet known. The sequence and exon/intron junctions in the coding region of the human and mouse genes are conserved; however, there is variability in both 5'- and 3'-UTRs, so that there is no direct correspondence of promoter regions in the two genes.

Tissue-, cell-, or differentiation-specific expression of cathepsin B might be due to the existence of several promoters with transcription factors acting differentially on each promoter and the level of those transcription factors dependent on cell type, tissue, stage of differentiation, and/or microenvironment. Recent studies in our laboratory on the putative promoter upstream of exon 1 in the human cathepsin B gene revealed a 20-fold enhancement in expression of a luciferase reporter gene by a segment of the gene >500 bp

upstream [55], suggesting that there is a positive regulatory element in that region. To our knowledge, this is the first evidence for transcriptional regulation of the human cathepsin B gene. We have also obtained evidence for post-transcriptional regulation of cathepsin B expression. In vitro transcription of a splice variant in which exon 1 is spliced to exon 3 revealed enhanced stability of this cathepsin B mRNA transcript as compared with the full-length transcript [56].

Increased expression of cathepsin B mRNA and protein correlates with tumor malignancy [2,20,21] (Table 2). In human colorectal carcinoma, cathepsin B mRNA levels are increased compared with matched normal colorectal tissues [57,58]. Immunohistochemical studies demonstrate that increased cathepsin B staining in colorectal carcinomas is predictive of shortened patient survival [59]. Another tumor in which cathepsin B expression is increased is human glioma [60]. Levels of cathepsin B transcripts are from three- to sixfold higher in low-grade astrocytoma and high-grade glioblastoma, respectively, than in normal brain. Increases in cathepsin B activity are even higher: In matched pairs of human brain tissues, cathepsin B activities are 10- and 15-fold higher in the glioblastoma tissues than in normal brain tissues [60]. The greater increase in cathepsin B activity in the gliomas does not appear to be due to a reduction in the endogenous cysteine protease inhibitors in gliomas [Rozhin,

Table 2. Comparative expression of cathepsin B in human cancers

	Breast	Colon	Glioma
mRNA ^a	nd ^b	4-fold	4-fold
Protein ^c	-/+	+++	+++
Activity ^d	8-fold	3-fold	12-fold
Secretion ^e			
Constitutive	+	+	nd
Inducible	+	+	nd
Intracellular distribution ^f	Focal adhesions	Basal/diffuse	Foot processes
Membrane association ^g	External surface	nd	nd
Prognostic value ^h	Yes	Yes	nd

^amRNA levels determined by northern blot analyses [58,60].

^bnd = not determined.

^cProtein levels determined by immunohistochemical staining [58,60,73].

^dActivities compared in tumor cells from invasive edges and in matched normal cells [68,69].

^eSecretion measured from cell lines: Constitutively secreted enzyme is procathepsin B and inducibly secreted enzyme is mature cathepsin B [90].

^fIntracellular distribution illustrated in Figure 3 or in Campo et al. [59].

^gMembrane association illustrated in Sameni et al. [92].

^hPrognostic value illustrated in a poster presented at the Keystone Symposium on Cancer Cell Invasion and Motility in Tamaron, Colorado (Lah TT, Kos J, Krasevec M, Golouh R, Vrhovec I, Turk V: Cathepsin B and L as possible prognostic factors in breast carcinoma) and in Campo et al. [59].

Any unattributed work is unpublished work from our own laboratory.

Mikkelsen, and Sloane, unpublished data], yet may reflect biological variability as mRNA levels were measured in unmatched samples and activities in matched samples. Levels of expression of cathepsin B (mRNA, protein, and activity) parallel the ability of glioblastoma cell lines to invade through Matrigel in vitro [61] and the invasive ability of gliomas in vivo as assessed by magnetic resonance imaging [60]. Thus, the studies on cathepsin B expression in human colon carcinomas and gliomas suggest that this enzyme plays a role in malignant progression of these two tumors.

As has been shown for other proteases [38,39,62-64], increases in expression of cathepsin B are not found uniformly throughout a tumor mass. Often these increases are found in the cells at the invading margins of a tumor, leading us to hypothesize that the expression of cathepsin B is upregulated in these cells in response to interactions between the tumor and surrounding stroma. Such increases in expression of cathepsin B mRNA and protein have been demonstrated at the invading front of bladder and prostate carcinomas by in situ hybridization and immunohistochemistry [65-67]. Furthermore, an inverse correlation between cathepsin B and type IV collagen staining is present at the invading edges of bladder tumors, suggesting that the increases in cathepsin B might be responsible for the local degradation of basement membrane [D.W. Visscher, M. Sameni, and B.F. Sloane, unpublished results].

These measurements of cathepsin B mRNA and protein do not tell us whether the amount of active cathepsin B is increased at the invasive edges, a prerequisite for cathepsin B to play a functional role in tumor growth and invasion. Therefore, we have measured cathepsin B activity in tumor cells isolated from the invasive edges using a microdissection technique. Tumor cells and matched normal epithelial cells have been microdissected from 10 μ m frozen sections of human colon tumors [68] and breast tumors [69]. Both cathepsin B and gelatinase A (MMP-2 or 72 kD type IV collagenase) activities are increased in the cells at the invasive edges. Since microdissection studies are labor intensive and not readily applicable to routine pathological analyses, we are attempting to develop monoclonal antibodies that can distinguish active forms of cathepsin B from the inactive pro forms. Such antibodies would be useful for immunohistochemical studies of a large number of samples from a wide variety of human tumors and, thus, for assessment of the importance of observed increases in expression of cathepsin B at the invading margins of human tumors.

Although it has been known since 1980 that breast tumor explants secrete increased amounts of cathepsin B [15], the levels of expression of cathepsin B in human breast cancer have not been as well characterized as those of cathepsin D. To our knowledge, studies on the expression of cathepsin B mRNA in breast cancer have not been performed. Increases in the expression of cathepsin B protein have been demonstrated by immunoassay (ELISA) of cytosolic extracts from breast cancer tissues [70]. Also, using an ELISA, Lah and coworkers [personal communication] have found that cathepsin B protein is 26-fold higher in cytosolic extracts of breast cancer tissue than in non-involved

adjacent tissues. Over a 5 year follow-up period, the patients with high levels of cathepsin B protein have a higher risk of recurrence and shortened overall survival. The increases in cathepsin B protein in breast cancer do result in increases in active cathepsin B because cathepsin B activity in breast cancer tissue is 10- to 20-fold higher than in matched pairs of adjacent normal tissues [70,71].

Furthermore, increases in cathepsin B activity are observed in breast cancer cell lines as well as in tissues [70-72]. In contrast, Castiglioni et al. [73] did not demonstrate an increase in staining for cathepsin B in human breast cancer, nor did they demonstrate an increase in staining for cathepsin D or for cathepsin L, another lysosomal cysteine protease. These apparent contradictory results may reflect regional heterogeneities in distribution of the cathepsins in tumors, a phenomenon also reported for other proteases [38,39,62-64]. In this regard, Castiglioni et al. [73] did not evaluate staining in invasive edges of the breast tumors, nor did they indicate whether there was increased staining in stromal cells. Thus, we were unable to assess either regional heterogeneities or the possible contribution of stromal cell cathepsin B to the measurements in tissue extracts of cathepsin B protein and activity [71]. Regional differences in cathepsin B in breast cancer do exist, because cathepsin B activity is eightfold higher in invasive tumor cells than in matched normal cells isolated by microdissection [69].

At present the mechanisms responsible for increases in the expression of cathepsin B in human tumors have not been fully elucidated. However, as discussed earlier, the increases in cathepsin B expression might be regulated transcriptionally and/or post-transcriptionally. In murine B16 melanoma, Qian et al. [74] observed that the transcription rate of the cathepsin B gene is increased and suggested that this is due to the presence of elevated levels of tumor-specific transcription factors. Although this same group found no evidence for a regulated promoter in the mouse cathepsin B gene [53], two other leader sequences are now known to be present [54]. Whether use of an alternative promoter is responsible for the increased rate of transcription in murine B16 melanoma has not been determined. Recent data from our laboratory suggests that the human cathepsin B gene also may have several promoters as well as an enhancer that can increase the expression of a luciferase reporter gene when transfected into a human glioblastoma cell line [55]. In some cells, expression of cathepsin B is dependent on their state of differentiation. For example, phorbol ester and granulocyte-macrophage colony-stimulating factor [75] and interleukin-1 [76] can induce an increase in expression of cathepsin B in parallel with the differentiation of promonocytes to macrophages or synovial fibroblasts, respectively.

Whether similar mechanisms are responsible for increases in expression of cathepsin B in tumors is unknown. Multiple transcript species have been detected in human tumors, arising from alternative splicing of the 5'- and 3'-UTRs [47-50,57,77,78], and therefore increases in expression of cathepsin B in human tumors might reflect changes in post-transcriptional processing. Tran-

scripts arising from splicing of exon 1 to exon 4 [47] or use of a putative alternative transcription initiation site before exon 4 [48] would encode a truncated form of procathepsin B lacking the signal peptide and part of the propeptide by using an in-frame AUG (methionine) codon in exon 4 to initiate translation. Gong et al. [47] proposed that transcripts missing exon 2 and 3 are tumor specific and that truncated procathepsin B contributes to the increase in cathepsin B expression in tumors. This group has expressed recombinant truncated procathepsin B by in vitro transcription/translation and by transfection in COS cells [79]. In addition to affecting the form of cathepsin B synthesized, alternative splicing of the 5'-UTR may result in mature mRNAs that vary in stability and/or translatability. Preliminary data on transcripts missing exon 2 indicate that they are more stable [56]. The presence of additional in-frame AUG codons followed by stop codons in exons 2, 2a, and 2b [48] suggests that transcripts missing these exons might be translated more efficiently. Clearly, more studies on the mechanisms of regulation of cathepsin B expression are needed to determine whether transcriptional and/or post-transcriptional mechanisms are responsible for the increases in cathepsin B expression in human tumors.

Altered trafficking of cathepsins D and B

Secretion

There is an extensive literature on the secretion of procathepsin D by breast cancers [18,19]. As early as 1980, Westley and Rochefort [17] reported that estrogen induces secretion of a 52kD protein by breast cancer cell lines; however, this protein was not identified as procathepsin D until 1986 [80]. Breast cancer cell lines secrete as much as 50% of their total cathepsin D in the form of procathepsin D: Secretion of procathepsin D by ER⁻ cells is constitutive, whereas that by ER⁺ breast cancer cell lines is induced by estrogen [18,19,35,81]. Mathieu et al. [82] postulated that the secretion of procathepsin D by breast cancer cells results from increased synthesis of cathepsin D overwhelming the mannose-6-phosphate receptors (MPRs) that would normally traffic procathepsin D from the Golgi to the late endosomes. Breast secretions from patients with breast cancer contain high levels of procathepsin D [83], indicating that procathepsin D is also secreted from breast cancer tissue in vivo. Nevertheless, the levels do not correlate with either the levels of procathepsin D in cytosolic extracts nor the estrogen receptor status of the breast cancer tissue.

Interestingly, procathepsin D is only 2–6% of the total cathepsin D in breast cancer extracts rather than the 50% observed in media of breast cancer cell lines [84]. Rochefort and colleagues have suggested that the lesser percentage of procathepsin D observed in cytosolic extracts reflects an in vivo activation of procathepsin D within the tumor. An alternative explanation is that the

larger percentage of procathepsin D found in breast cancer cells in culture is due to modifications in trafficking of this enzyme induced by culture conditions. If procathepsin D is secreted *in vivo*, we do not know whether it is secreted from breast cancer cells or stromal cells. We also do not know whether secreted procathepsin D would be activated extracellularly or intracellularly following its endocytosis by cancer cells or stromal cells. Because activation of procathepsin D requires an acidic pH [85], activation most likely occurs intracellularly in an acidic compartment such as lysosomes or endosomes, perhaps including the large acidic vesicles identified by Rochefort and colleagues in breast cancer cells [86].

Cathepsin B is also secreted by tumors, but often not to the same extent as is cathepsin D [2,20,21]. An early report on secretion of cathepsin B from breast cancer explants did not distinguish between procathepsin B and mature cathepsin B [15]. Increases in the secretion of procathepsin B by human colorectal carcinoma cell lines [87] and hepatomas [88], as well as by human breast cancer explants and cell lines [15,16], have been reported. Secretion of procathepsin B, in apparent contrast to that of procathepsin D, can occur from cell that do not exhibit an increase in mRNA levels for cathepsin B [E. Friedman, M. Ahram, and B.F. Sloane, unpublished data; 89]. Secretion of mature cathepsin B also occurs from human colorectal tumor cells as well as that of procathepsin B [87]. In *ras*-transformed MCF-10A1neoT cells and murine melanoma and human colorectal carcinoma cell lines, we have shown that secretion of mature cathepsin B can be induced, whereas the secretion of procathepsin B occurs constitutively [90,91] (Table 2). We hypothesize that secretion of procathepsin B occurs via the default pathway and that of mature cathepsin B from an endosomal/lysosomal compartment that is readily mobilized in tumor cells and transformed cells [89,90,92]. A readily mobilized endosomal/lysosomal compartment has been observed in macrophages [93], suggesting that this may be common to the cells that participate in local degradation of extracellular matrices.

Membrane association

In macrophages [94] and hepatocytes [95], endosomes have been observed to contain cathepsin D. Furthermore, Stahl and colleagues [96] have shown that cathepsin D is associated with the membrane of macrophage endosomes. The form of cathepsin D associated with the membrane is the single chain mature form. In breast cancer cells, endosomes have also been shown to contain mature cathepsin D, as demonstrated by double immunofluorescence studies using markers for endocytosis and a monoclonal antibody specific for mature cathepsin D. The endosomal vesicles in this case are large acidic vesicles rather than small vesicles and are heterogeneous in nature. Montcourrier et al. [86] postulate that the function of mature cathepsin D in these large acidic vesicles is the digestion of phagocytosed and/or endocytosed extracellular matrix and consequently the facilitation of tumor cell invasion and metastasis.

In human and animal tumors, tumor cells, and transformed cells, we have consistently found that cathepsin B can be sedimented with a subcellular fraction enriched in plasma membrane and endosomes as well as a subcellular fraction enriched in lysosomes [2,20,21]. Only mature forms of cathepsin B are found in the membrane fractions as assessed by attempting to increase the activity by incubation with pepsin at acidic pH [89,97,99], a procedure that will activate procathepsin B in vitro. That only mature cathepsin B is found in the membrane fractions has been further confirmed by immunoblotting with cathepsin B antibodies [89] and by purifying cathepsin B from these fractions [98].

The presence of active cathepsin B in membrane fractions of tumors coincides with cytochemical observations of cathepsin B activity at the surface of tumor cells [21,99]. For example, in cytochemical studies on malignant breast cancer cells, a microgranular staining pattern indicative of cathepsin B activity is seen both in the perinuclear region (presumably in lysosomes) and throughout the cytoplasm, including in cytoplasmic projections at the cell surface [100]. A similar pattern has also been observed in highly metastatic B16 amelanotic melanoma cells and, in this case, the staining for cathepsin B activity has been shown to be abolished in the presence of E-64, an irreversible inhibitor of cysteine proteases [21,99] (see Table 1). Studies on the localization of cathepsin B protein in tumor cells and tissues confirm the cytochemical observations on the distribution of cathepsin B activity in tumor cells. In a number of human tumors [59,60,67,101,102], including human breast carcinoma [92], cathepsin B protein has been localized to the basolateral region of the cells, to regions adjacent to the cell membrane, to cell processes, and to the cell membrane itself. Immunofluorescent and immunogold studies have established that cathepsin B becomes associated with the cell membrane and cytoplasmic projections of MCF-10A1 human breast epithelial cell subsequent to their transfection with the *c-Ha-ras* oncogene [103-105] (also see chapter by Miller et al. for a further description of these cell lines). Cathepsin D undergoes a similar change in localization [92]. Thus, membrane association of cathepsins B and D occurs at an early stage of malignant progression of breast epithelial cells.

Since cathepsin D and cathepsin B are both lysosomal enzymes, the altered distribution of the two enzymes in *ras*-transformed MCF-10A1neoT cells may simply reflect an altered distribution of the lysosomal compartment or an endosomal compartment. To test this hypothesis, we have performed double-labeling studies for the two enzymes. In parental MCF-10A1 cells, that is, a diploid immortal cell line, cathepsin D and cathepsin B colocalize in perinuclear vesicles [92]. In the *ras*-transformed MCF-10A1neoT cells, perinuclear vesicles label for both cathepsin D and B, and peripheral vesicles label for cathepsin D alone or cathepsin B alone [92]. The fact that the two cathepsins are distributed in different vesicular compartments in transformed cells and tumor cells may be indicative of functional differences.

The presence of cathepsins D and B in distinct vesicles also suggests that the

two cathepsins may be trafficked via different pathways [6,7]. Whether a similar mechanism might also be responsible for the membrane association of cathepsin B and cathepsin D is under investigation. Lysosomal enzymes are targeted to lysosomes primarily via the MPR pathway [5]. Both cathepsin D [82] and cathepsin B [106] can be trafficked via the MPR pathway, but whether this pathway is responsible for all of the trafficking of the two cathepsins is not clear. An alternative trafficking pathway, the mannose receptor (MR) pathway, has been identified in macrophages and has been shown to be involved in trafficking of cathepsin D [6].

McIntyre and Erickson [107] have proposed that proforms of two lysosomal proteases, cathepsins D and L, are targeted to the lysosome via two sequential mechanisms: The first is dependent on N-linked carbohydrates (e.g., MPRs or MRs) and the second on a 'targeting signal' or 'proenzyme receptor' binding region in the protein sequence. A lysosomal proenzyme receptor (LPR) responsible for trafficking of procathepsin L has been identified [7]. Mannose 6-phosphate-independent pathways have been identified in macrophages [96] and HepG2 cells, a hepatoma cell line [108]. At this time, Erickson and colleagues have not shown that procathepsin B can be trafficked via the LPR pathway.

Defects in MPRs (an absence or an increased rate of turnover) have been reported in tumor cell lines [109]. A decrease in the number of MPRs or the affinity of MPRs on the cell surface of virally transformed 3T3 fibroblasts has been suggested to be responsible for the secretion of procathepsin B by these cells [110]. Takeshima et al. [111] have shown that in hepatocytes unglycosylated procathepsin D synthesized in the presence of tunicamycin is correctly transported to the lysosomes and is processed to the mature enzyme. However, a deletion mutant of cathepsin D lacking the propeptide, although glycosylated, appears to be accumulated in the endoplasmic reticulum. These observations would suggest that the trafficking of cathepsin D in hepatocytes does not involve either MPRs or MRs, but rather involves a receptor such as the LPR that recognizes the propeptide of cathepsin D. The pathways responsible for targeting procathepsins D and B to lysosomes in normal cells need to be better understood before we can unravel the possible modification in the trafficking of these enzymes to lysosomes in transformed and tumor cells, and how this leads to compartmentalization of cathepsins D and B in distinct vesicles and membrane association of the two cathepsins.

Is cathepsin D a prognostic marker for breast cancer?

Human breast cancers are the leading cause of cancer deaths for women in the United States. Therefore, identifying prognostic factors that will distinguish women at risk for early relapse and metastasis is a critically important goal. Rochefort's group has, for a long time, observed a consistent correlation between cathepsin D and prognosis in patients with breast cancer

Table 3. Prognostic value of cathepsin D in breast cancer cytosols

No. patients	Shortened survival	High risk of relapse	Other information	Ref.
122	+	+	Positive staining correlates with better prognosis, most evident in ER ⁺ or node-positive subsets	112
396	nd	nd	Shorter relapse-free survival in premenopausal and postmenopausal patients	113
140	nd	nd	Independent of <i>neu/erbB2</i> and <i>int-2</i> ; correlates with <i>c-myc</i>	84
397	+	+	Node-negative patients only	34
413	+	+	Shorter survival in node-positive patients	117
139	+	+	All in node-negative patients	116
331	+	nd	Only in ER ⁻ subgroup	119
123	+	+	Only prospective study to date; correlates with axillary lymph node involvement	118

nd = not determined.

[36,81,113,114]. Others, relying primarily on immunohistochemical techniques, have not confirmed this [73,115].

Levels of cathepsin D in cytosolic extracts of breast tissue are measured most frequently by a sandwich ELISA, which uses two monoclonal antibodies. One of these recognizes the 34 kD form of mature cathepsin D, and the other recognizes the 48 and 52 kD proforms of cathepsin D (see Table 1) [113,116]. This assay is simple and reliable; furthermore, >90% of all forms of cathepsin D have been shown to be extracted by the homogenization procedure used to prepare cytosolic extracts of breast tissue. The findings in terms of prognostic value of cathepsin D ELISAs are summarized in Table 3.

In Cox multivariate studies, cathepsin D is one of the three most significant prognostic markers for breast cancer [37,117]. In both retrospective [84,113,114,118] and prospective [119] studies, a high cytosolic level of cathepsin D in breast cancer is associated with shortened patient survival and a higher risk of early relapse and metastasis. If patients are divided into specific subgroups, the published studies support the contention that high cytosolic levels of cathepsin D indicate a poor prognosis for breast cancer patients. A high cytosolic level of cathepsin D does not seem to be related to other important prognostic factors for breast cancer, such as degree of lymph node invasiveness, tumor size, Scarff and Bloom histological grade, DNA ploidy, patient age [34,84,113,114,117,118,120], or amplification of the *neu/erbB2* or *int-2* oncogenes [84]. Cathepsin D levels correlate with ER status only in premenopausal patients [114], which is consistent with the high constitutive production of cathepsin D by ER⁻ cell lines. The prognostic value of cathepsin D, therefore, supplements that of other markers. The independence of cathepsin D from other markers suggests that cathepsin D is associated with a stage

of breast cancer progression that differs from that detected by other prognostic parameters of breast cancer.

An important limitation of ELISAs is that neither the cellular origin (cancer cells, macrophages, or fibroblasts) nor the subcellular localization of cathepsin D is determined. Therefore, investigators have performed immunohistochemical analyses of cathepsin D in breast tissues. In contrast to ELISAs, the prognostic value of cathepsin D staining has been found to be negative in some cases and positive in others (Table 4). Although Henry et al. [114] suggest that the difference between the ELISA and immunohistochemistry results may reflect secreted procathepsin D that cannot be determined by immunohistochemistry, this would seem unlikely because the contribution of procathepsin D to total cathepsin D level is generally low (<6%) [84]. Even immunohistochemical studies using the same antibody give contradictory results: Positive staining for cathepsin D indicates a better prognosis in one study [115], yet intense cathepsin D staining is associated with shortened patient survival in two other studies [32,121].

The current controversy over the value of cathepsin D as a prognostic marker in breast cancer is highlighted by two recent independent studies. Castiglioni et al. [73], using a rabbit antiserum to purified human cathepsin D from Dako (Carpenteria, CA), found no evidence for cathepsin D being of prognostic value in breast cancer. In contrast, Roger et al. [36], using the M1G8 mouse monoclonal antibody from Biosys (Gif-sur-Yvette, France) that recognizes procathepsin D, found that immunohistochemical staining for cathepsin D confirmed their ELISAs showing that cathepsin D is an independent prognostic marker for breast cancer. These contradictory results might arise from the fact that, in addition to tumor cells, stromal cells (mainly macrophages and lymphocytes) within breast cancer tissue can express cathepsin D [36,41,121-123]. A further consideration in the immunohistochemical analyses is the distribution within the tumor of the section being stained for cathepsin D (or any other potential prognostic marker). In this regard, we have shown that for cathepsin B, cells at the invasive edges of bladder [65] and prostate [66,67] tumors stain intensely, whereas cells further back in the tumor mass may exhibit no staining. Analyses of cathepsin B activity in tumor cells microdissected from the invasive edges of colorectal [68] and breast [69] cancers also reveal an increased expression of cathepsin B in these regions. Whether the observations on cathepsin B will extend to cathepsin D is not known.

Several recent studies have reported that proteases, including cathepsin D, are present in stromal cells of malignant tumors, suggesting that stromal cells may participate in the process of invasion [122-125]. Unfortunately, many studies, including that of Castiglioni et al. [73], have not provided an independent evaluation of the prognostic importance of tumor cell and stromal cell cathepsin D expression in breast cancer [32,73,120,123]. Visscher et al. [41] have established that cathepsin D staining of stromal fibroblasts, but not of tumor cells, shows a significant correlation with metastasis in breast cancer

Table 4. Immunohistochemical staining for cathepsin D in breast cancer

No. patients	Prognostic value	Antibody ^a	Tumor cells	Stromal cells	Other information	Ref.
103	+	Novocastra polyclonal IgG	69% (criterion of >25% positive cells)	73% (criterion of >25% positive cells)	Increased stromal cell (but not tumor cell) staining correlates with tumor grade and shortened patient survival	121
136	-	Novocastra polyclonal antiserum	60% (criterion of many granules in majority of cells)	73% (criterion of many granules in majority of cells)	No correlation between staining of tumor cells and survival at 5 years	123
245	nd ^b	Dako polyclonal antiserum	49% ^c (staining detectable)	nd	Not an independent marker of survival	32
80	-	Dako polyclonal antiserum	90% (staining detectable)	nd	No correlation between staining and survival or other aggressive behavior	73
562	-	Biosys monoclonal M1G8#	nd	nd	77% positive staining of frozen sections; no relationship between staining and survival	127
41	+	Biosys monoclonal M1G8#	QIC score ^d 65 (2-373)	QIC score ^d 79 (2-220)	Staining intensity in tumor cells, but not macrophages, ^e useful in evaluating risk of metastasis	36
359	+	Polyclonal antiserum	74% (granular cytoplasmic staining detectable)	nd	Correlates with positive nodes; not independent of other tumor variables	120
213	+	Monoclonal 1C11 (IgG1)	77% (staining detectable)	76% (staining detectable)	High stromal cell, but not tumor cell, staining associated with poor survival	122
86	+	Biosys monoclonal	70% (staining detectable)	89% (staining detectable)	Stromal cell staining correlates with positive nodes and metastasis; sum of tumor and stromal cell staining correlates with shortened survival	41

^aAntibodies in references 121 and 123, references 32 and 73, and references 36 and 127 were from the same origin. The antibodies used in other studies were either prepared in the authors' laboratories [120] or not further described in the publications.

^bnd = not determined.

^cHistoscore combining intensity and distribution of staining with the estimated proportion of cells stained.

^dQIC = computerized quantitative immunohistochemical score (% of stained surface \times mean staining intensity $\times 10 \times$). QIC scores listed from ductal breast carcinoma only.

^eMacrophages were stained with an anti-CD68 monoclonal antibody (Dakopatts, Glostrup, Denmark).

patients. Recently, two more reports [122,123], using yet another two separate antibodies to cathepsin D (Table 4), demonstrated that increased stromal cell cathepsin D staining is associated with shortened patient survival, but increased tumor cell staining is not. Roger et al. [36] found that macrophages stain heavily for cathepsin D, particularly macrophages in the tumor periphery. In this case, an anti-CD68 monoclonal antibody was used to identify macrophages. Nevertheless, the cytosolic levels of cathepsin D correlate with cathepsin D expression in cancer cells rather than with the number of macrophages.

In another independent study, Isola et al. [37] evaluated cathepsin D expression in macrophages and cancer cells in 262 node-negative breast cancers. In this study, cathepsin D staining in cancer cells, rather than in macrophages, correlates with shortened patient survival. Most studies support the hypothesis that increased expression of cathepsin D in breast cancer cells, rather than the recruitment of macrophages that express cathepsin D, is responsible for the high risk of relapse and development of metastases. This is further substantiated by the increased metastatic capability of tumor cells that overexpress cathepsin D due to transfection with a cathepsin D cDNA [126].

The conflicting results of Castiglioni et al. [73] and Rogers et al. [36] might also be due to the use of different antibodies. Antibodies can differ in their affinity. In addition, as the cathepsin D molecule exists in at least three different protein forms, each of which may differ in glycosylation, antibodies may recognize distinct molecular configurations of the same molecule. However, differences in affinities for cathepsin D and in the specific forms of cathepsin D recognized cannot explain the disparate results obtained in studies using the same polyclonal [121,123] or monoclonal antibody [36,127]. Another possibility is that the use of different fixation procedures and/or methods for quantitation of cathepsin D staining in breast cancer tissue leads to contradictory results (Table 4). Perhaps, given the lack of uniformity in the assays used, contradictory results should be expected. More large-scale cooperative studies in which the pathologists use the same antibody and fixation procedures, as well as the same methods for quantitation, are needed to determine the true prognostic value of cathepsin D staining [121]. Studies using multiple techniques that might provide an internal confirmation of positive or negative findings would also be of value. In such a recent study, Ravdin et al. [127] performed immunohistochemical staining for cathepsin D, but could not confirm their earlier findings by immunoblotting that cathepsin D is a prognostic marker in node-negative breast cancer.

Review of the clinical studies on cathepsin D leads one to the conclusion that the prognostic significance of cathepsin D determinations, although provocative, are still investigational in nature and not yet ready for widespread clinical application. The assays (whether ELISAs, immunohistochemical, or immunoblotting) need to be standardized to ensure quality control. This, together with a deeper understanding of the patient subsets, will be required to fully define the role of cathepsin D in breast cancer.

Role of cathepsin D and cathepsin B in breast cancer progression

Both clinical and basic studies on expression of cathepsins D and B in breast cancer indicate that high cathepsin levels are either an epiphenomenon of malignant progression or, more excitingly, play a direct role in at least one step of malignant progression. Several reports have confirmed that increased expression of cathepsins D and B (mRNA, protein and activity) is a marker of the malignant phenotype [2,18,59,60]. Direct evidence that overexpression of cathepsin D in breast cancer cells can promote some steps in metastasis has been obtained in adenovirus-transformed 3Y1 normal rat embryo cells transfected with a cathepsin D cDNA [126].

The mechanisms by which cathepsins are involved in tumor invasion and progression are not clear. Cathepsin B may facilitate invasion directly by degrading extracellular matrix [10] or indirectly by activating other proteases, such as urokinase [12]. Increased expression of cathepsin B at the basolateral surface of colorectal carcinoma cells [59], that is, the surface in direct contact with basement membrane, suggests that this enzyme may be involved in tumor invasion. A strong correlation between overexpression of cathepsin B and a shortened patient survival of colorectal carcinoma indicates that cathepsin B may be a significant prognostic indicator. Briozzo et al. [128] established that the degradation at acidic pH of extracellular matrix by proteases secreted into the conditioned media of breast cancer cells is due primarily to cathepsin D, since it can be completely inhibited by pepstatin, an aspartic protease inhibitor (Table 1). Secreted cathepsin D might also promote the spread of breast cancer by activating other proteases, including procathepsin B or degrading extracellular matrix proteins [21,81,129]. Nevertheless, the pH optimum for cathepsin D is so acidic that a significant extracellular role for this enzyme seems unlikely. Cathepsin D may play a role in digestion of extracellular matrix proteins intracellularly: Matrix proteins have been shown to be degraded by cathepsin D within large acidic vesicles in breast cancer cells [86], presumably following internalization by phagocytosis.

Proteases located on the external cell surface of breast cancer cells might be responsible for extracellular matrix degradation. For cathepsin B, we have shown that the membrane-associated enzyme is a mature active form [89]. Immunofluorescence and immunogold studies indicate that cathepsin B [92] and cathepsin D (Figures 2 and 3) are concentrated on the external surface of the plasma membrane. The localization of these enzymes on the surface of human breast epithelial cells is increased by malignant progression (see Figures 2 and 3; also see Figure 5 in Sameni et al. [92]). Three-dimensional reconstructions of optical sections taken through the cells indicate that in *ras*-transformed MCF-10A1neoT cells cathepsin D is localized primarily on the basolateral surface of the cells (Figure 3), as is cathepsin B [130]. We have previously shown that cathepsin B is also on the basolateral surface of MCF-7 and BT20 human breast carcinoma cell lines [92].

Recent studies indicate that cathepsin B is concentrated intracellularly in

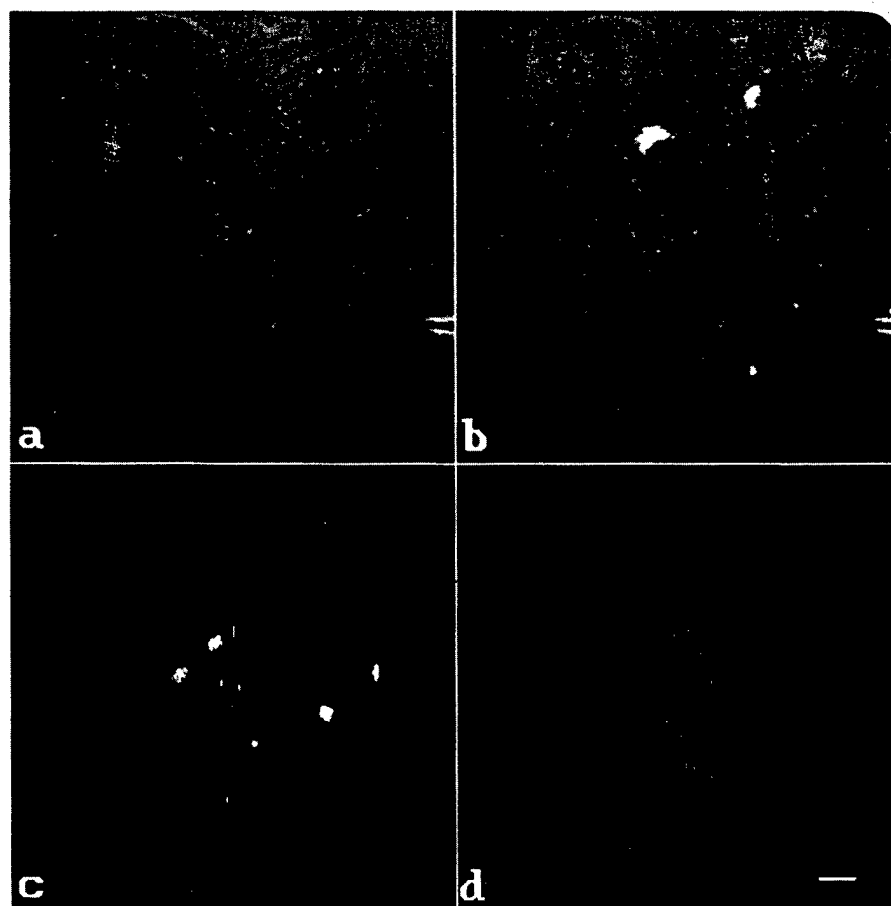


Figure 2. Only a few discrete areas on the external surface of parental MCF-10A human breast epithelial cells exhibit fluorescent labeling for cathepsin D. **a:** Phase contrast image of MCF-10A cells. **b:** Single optical slice of fluorescent labeling for cathepsin D that has been superimposed on the phase contrast image of panel a. **c:** Three-dimensional reconstruction of optical slices of fluorescent labeling for cathepsin D. **d:** Control in which the primary antibody was replaced with nonimmune serum. The primary antibody was rabbit anti-human cathepsin B IgG [52,89], and the secondary antibody was Texas red-conjugated donkey anti-rabbit IgG. Bar, 10 μ m.

regions of the cells shown to be involved in matrix degradation (Table 2). Treatment of MCF-7 breast carcinoma cells with tumor necrosis factor α results in the concentration of cathepsin B staining at focal adhesions. In a highly malignant human glioblastoma cell line derived from a glioblastoma that expresses 15-fold more cathepsin B activity than matched normal brain tissue, cathepsin B is concentrated in foot processes. These foot processes resemble invadopodia, highly specialized matrix-degrading structures described by Monsky et al. [131]. Other proteases have been localized to invadopodia [132]. Perhaps this facilitates the proteolytic cascade responsible

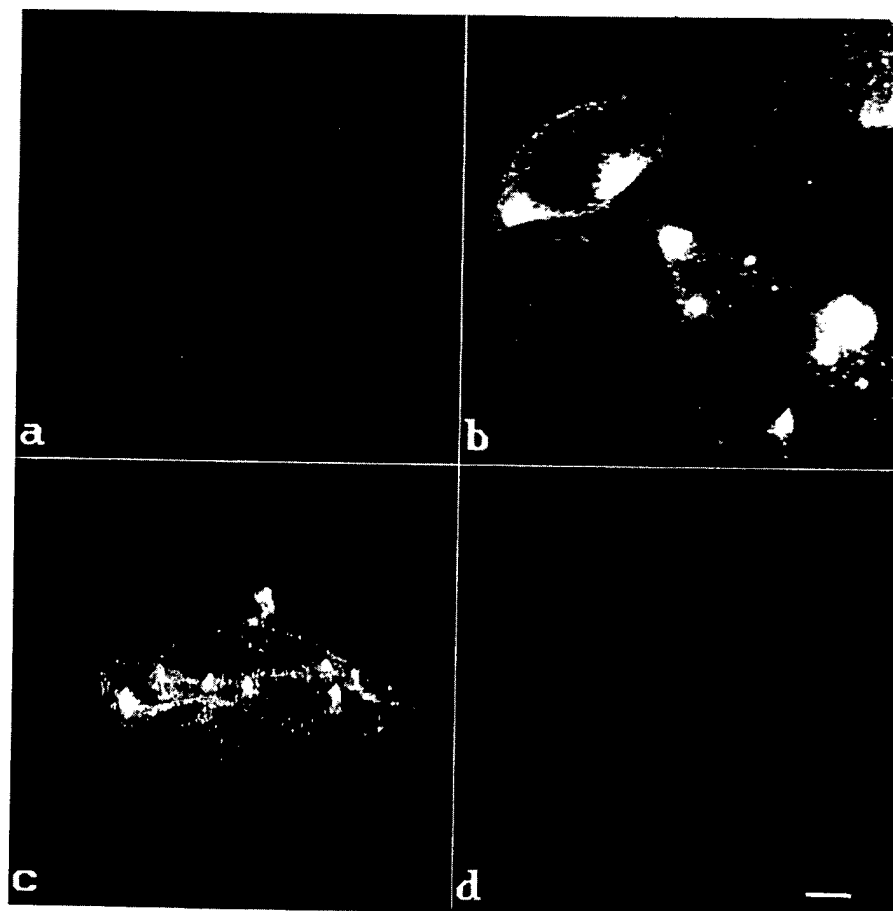


Figure 3. *Ras*-transformation of the MCF-10 human breast epithelial cells resulted in a large increase in labeling for cathepsin D on the external basolateral surface of these cells. **a:** Phase contrast image of MCF-10AneoT cells. **b:** Single optical slice of fluorescent labeling for cathepsin D that has been superimposed on phase contrast image of panel a. **c:** Three-dimensional reconstruction of optical slices of fluorescent labeling for cathepsin D. **d:** Control in which the primary antibody was replaced with nonimmune serum. The primary antibody was rabbit anti-human cathepsin B IgG [52,89], and the secondary antibody was Texas red-conjugated donkey anti-rabbit IgG. Bar, 10 μ m.

for degradation of extracellular matrix by invading cells. Many membrane-associated proteases have been identified in breast cancer; these include gelatinase A (MMP-2 or 72 kD type IV collagenase) and its putative membrane receptor [131], stromelysin 3 [133], urokinase and its receptor [134], and cathepsins B and D [89,92]. The localization of proteases on the cell surface will result in an increase in their local concentration to a point that the balance between proteases and their endogenous inhibitors will favor proteolysis [135], thus favoring the degradation of basement membrane and tumor cell

invasion. The accessibility of proteases at the surface of tumor cells might be exploited for diagnostic or therapeutic purposes, for example, by the development of specific antibodies to identify micrometastases, by the development of specific antibodies and inhibitors that can block the activities of the proteases, and by the development of specific antibodies that can target cytotoxic agents to kill the targeted cells.

Another possible function for procathepsin D is to promote the proliferation of breast cancer cells by acting as an autocrine mitogen similar to insulin-like growth factor II (IGF II) [136,137]. However, in contrast to IGF II, procathepsin D has a more pronounced mitogenic effect on breast cancer cell lines; proliferation can be inhibited by antibodies specific for the propeptide of cathepsin D [137]. This suggests a specific function for procathepsin D in the progression of breast cancer. The mechanisms for this mitogenic activity of cathepsin D are unknown. However, recent observations suggest that this enzyme may induce cell proliferation by activating latent forms of growth factors or by interacting with growth factor receptors extracellularly or intracellularly [137]. Another possibility is that secreted cathepsin D from breast cancer cells upregulates the activity of IGF by degrading IGF-binding protein-3, a protein that normally binds to IGF peptides and modulates their biological activities [138].

Studies to date support, but do not prove, that cathepsins D and B play functional roles in malignant progression. In breast cancer, cathepsin D appears to play a leading role in progression and metastasis, as indicated by the positive correlation between the level of cathepsin D (protein and activity) and malignant progression, and the high risk of early relapse and metastasis observed in patients with breast cancer [34,84,114]. An important functional role for cathepsin D in breast cancer is further supported by the fact that tumor cells transfected with a mammalian expression vector of human cathepsin D exhibit an increased metastatic potential [126]. In other cancers, for example, in melanoma [47,139,140] and gliomas [60], cathepsin B appears to play a more important role than does cathepsin D. These observations, as well as studies on the roles of metalloproteases and serine proteases in breast cancer and other cancers, suggest that more than one protease is required for malignant progression. The proteases involved appear to depend on the tissue of origin of the cancer. Furthermore, the functional proteases may change depending on the stage of malignant progression. Understanding the roles played by cathepsins D and B and by other proteases will require cooperation among investigators, as we need to evaluate multiple proteases concurrently in a cancer arising in a single tissue, such as breast cancer, and at various stages during the progression of that cancer.

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Is Altered Localization of Cathepsin B Causally Related to Malignant Progression?

Jennifer E. Koblinski† and Bonnie F. Sloane†*

†Cancer Biology Program and *Department of Pharmacology,
Wayne State University, School of Medicine, Detroit, MI 48201 USA

Abstract. Increased expression of cathepsin B parallels malignant progression in human colon carcinoma and glioblastoma and most likely other tumors. The invasive edges of human bladder, breast, colon and prostate carcinomas and the infiltrating regions of glioblastoma exhibit particularly high expression of cathepsin B at the mRNA, protein and activity levels, suggesting that tumor/stromal interactions may regulate expression of cathepsin B. Altered localization of cathepsin B within and on the surface of tumor cells is a distinctive and recurring feature during progression of human tumors. Cathepsin B staining is localized to the inner and outer aspects of the basal surface of the tumor cells. The localization of cathepsin B at the cell surface and specifically at focal adhesions may provide clues to the function of this protease in tumor cell invasion. Cathepsin B does participate in tumor cell invasion as inhibitors of cathepsin B can reduce invasion in both *in vitro* and *in vivo* assays.

1. Introduction

1.1 Involvement of proteases in tumor cell invasion

Tumor cell invasion involves attachment of tumor cells to the underlying basement membrane, local proteolysis and migration of tumor cells through the proteolytically modified region [1]. Local proteolysis is facilitated by proteases outside the tumor cell, perhaps bound to the cell surface, and/or secreted from the tumor cell (figure 1). Increasingly, data suggest that proteases may be involved in local proteolysis via two mechanisms: i). directly by degradation of the extracellular matrix or ii). indirectly by activating other proteases which will degrade the extracellular matrix. Tumor cells, stromal cells, and fibroblasts have been shown to play a role in this process. Fibroblasts may secrete proteases which digest the surrounding matrix in a soluble form or bind to tumor cells and thereby digest the extracellular matrix. Fibroblasts may send a message to the tumor cells that leads to synthesis of proteases and/or protease inhibitors by the tumor cells or the tumor cells may induce production of proteases and protease inhibitors by stromal cells. Tumor/stromal interactions are very complex and have not been completely resolved.

Proteases from the aspartic (e.g., cathepsin D), cysteine [e.g., cathepsin B (CB) and cathepsin L], metallo- (e.g., gelatinases A and B) and serine (e.g., urokinase plasminogen activator) classes have been linked to tumor progression and invasion. Expression of the

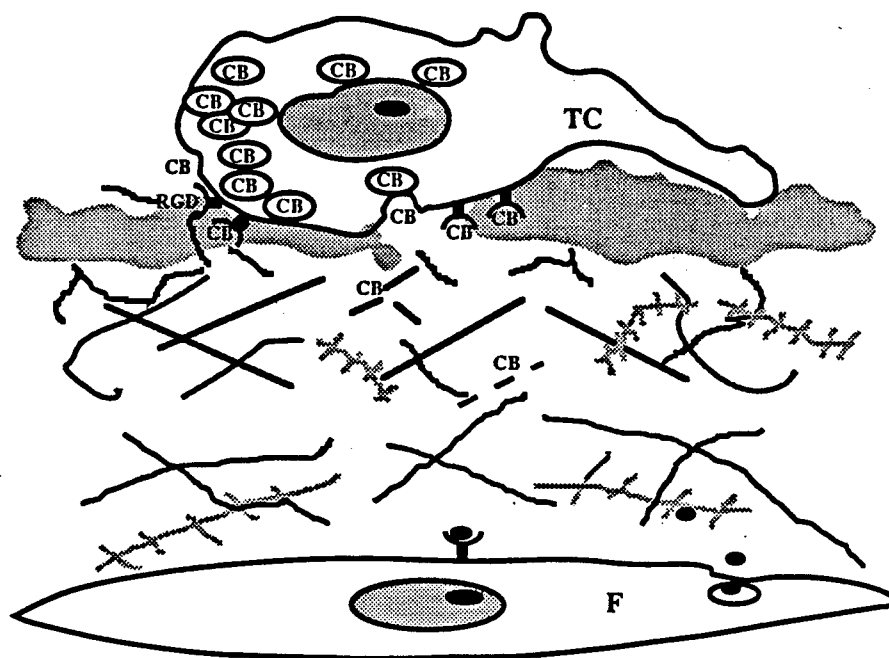


Figure 1. Schematic drawing representing localized proteolysis. Proteases involved in matrix degradation appear to be secreted from tumor cells (TC) and surrounding stromal cells such as fibroblasts (F). The secreted proteases may bind to receptors on the cell surface thereby localizing proteolysis to specific regions. Interactions between tumor cells and fibroblasts often induce increased expression of proteases. In this cartoon, we illustrate secretion of CB from tumor cells and its binding to the cell surface.

lysosomal cysteine protease, CB, increases in parallel with malignant progression of a wide variety of human tumors. Human bladder, breast, cervical, colon, esophageal, liver, lung, pancreatic, rectal, stomach, and ovarian carcinomas have all been found to overexpress CB [for review see 2].

1.2. Regulation of CB

CB has broad endopeptidase activity as well as carboxydipeptidase activity [3,4]. This suggests that there are many natural substrates for this protease. CB degrades both synthetic and protein substrates over a range of pHs (pH 5 to 8) [5-8]. Larger protein substrates, e.g., extracellular matrix components, can stabilize the enzyme *in vivo* so that it is active at neutral pH [7]. CB can directly degrade the basement membrane proteins laminin, fibronectin, and collagen IV [7,9,10] and can activate other proteases such as prourokinase plasminogen activator and procollagenase which have been shown to degrade the extracellular matrix [11-13].

The location of CB may be a clue to its pathological function. If this enzyme is to participate in local proteolysis, one would expect it to be localized at or near the cell

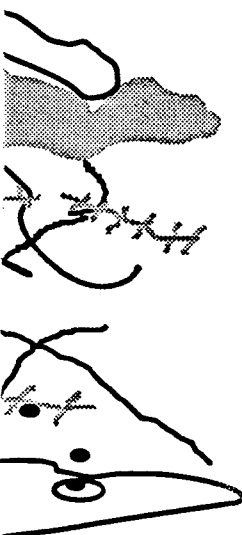
periphery, secreted from preproenzyme with the reticulum. In the phosphomannosyl residue dependent and independent environment of the late endosome, the form is cleaved with the form and in some tissues a light chain of 5 kD [8] region and in other yet also found on the cell surface suggest that CB can be extracellular matrix. The glycosylation state or site explain this. A lysosomal receptor, which specifically have shown that procath

The expression posttranscriptional, promoter for CB is located on chromosome of heterozygosity has been untranslated regions and [19]. These transcripts rates resulting in altered have a deletion of the promoter this case a truncated CB trafficked to the cytoplasm. Transcripts lacking exon those initiated at a putative choices of promoters and expression of CB. For example determined to target of Whether this will be the

2. Cathepsin B Expression

2.1. CB expression: A potential

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periphery, secreted from the cell or associated with the cell surface. CB is synthesized as a proenzyme with the prepeptide directing the enzyme to the lumen of the endoplasmic reticulum. In the endoplasmic reticulum and Golgi network, CB acquires phosphomannosyl residues. These residues target CB to the lysosomes via cation dependent and independent mannose 6-phosphate receptors [14-17]. The acidic environment of the late endosome/early lysosome leads to dissociation of the proenzyme from the mannose 6-phosphate receptor. The propeptide of the inactive 43 kD/46 kD form is cleaved with the processing resulting initially in an active 31 kD mature single chain form and in some tissues in an active double chain form with a heavy chain of 25/26 kD and a light chain of 5 kD [8]. In cancer cells, CB is localized in lysosomes in the perinuclear region and in other yet unidentified vesicular compartments at the cell periphery. CB is also found on the cell surface and is secreted from cancer cells (figure 1). These results suggest that CB can be mobilized to cell compartments or membranes adjacent to the extracellular matrix. The reason for this alternate trafficking is presently unknown. The glycosylation state or saturation of the mannose 6-phosphate receptor does not fully explain this. A lysosomal proenzyme receptor, i.e., an alternative microsomal membrane receptor, which specifically binds procathepsin B has not been identified. McIntyre *et al.* have shown that procathepsin L can be trafficked by such a receptor [18].

The expression and activity of CB can be regulated at transcriptional, posttranscriptional, processing, trafficking and inhibitor levels [for review see 2]. The gene for CB is located on chromosome 8p22. In some cancers, particularly prostate cancer, loss of heterozygosity has been identified in this region. Transcript variants involving 5' and 3' untranslated regions and the 5' coding region of CB have been identified in human tumors [19]. These transcript variants could have different stabilities and/or different translation rates resulting in altered expression of CB. In particular, transcripts lacking exon 3 would have a deletion of the prepeptide and part of the propeptide protein-coding sequence. In this case a truncated CB protein would be synthesized that would be expected to be trafficked to the cytoplasmic compartment due to the absence of a signal peptide. Transcripts lacking exon 3 could occur from those spliced between exons 1 and 4 [20] or those initiated at a putative alternative promoter in front of exon 4 [19]. Alternative choices of promoters and initiation sites might affect the localization as well as the levels of expression of CB. For example, alternative promoter usage and initiation codons have been determined to target of oligopeptidase M to different subcellular compartments [21]. Whether this will be the case for CB will need to be determined.

2. Cathepsin B Expression in Tumor Tissue

2.1. CB expression: A prognostic indicator in colon carcinoma and glioma

Increases in mRNA levels, protein staining and activity of CB have been found in many types of tumors [for review see 2]. In colon [22,23] and gastric [W.P. Ren and B.F. Sloane, unpublished results] carcinomas and glioblastomas [24], mRNA levels of CB are increased in tumors as compared to matched normal tissue. In both colon carcinomas and

Table 1. Correlation between CB Expression in Colon Tumor Cells and Dukes Stage [25].

Dukes Stage	Number of Tumors	No Staining	Low Staining	High Staining
A	8	5 (62%)	3 (38%)	0 (0%)
B	20	1 (5%)	14 (70%)	5 (25%)
C	30	3 (10%)	14 (46%)	13 (44%)
D	11	0 (0%)	3 (28%)	8 (72%)

glioblastomas, further increases parallel malignant progression. Protein staining for CB is also increased in parallel with progression of colon carcinoma [25] (table 1) and glioblastoma [25,26]. In colon carcinoma, high levels of CB staining in tumor cells is associated with significantly ($p < 0.001$) shorter overall patient survival [25]. In normal brain and low-grade astrocytomas, little staining for CB is observed, whereas more extensive staining is seen in anaplastic astrocytomas. In glioblastomas, an intense granular staining pattern is observed for CB in tumor cells [24,26]. CB staining is not seen in the central necrotic region of glioblastomas, thus indicating that the CB activity measured in tissue homogenates emanates from viable tumor and/or normal brain cells [27]. Others have observed similar staining patterns for CB in glioblastomas [28]. Rempel *et al.* [24] correlated increases in CB protein in human gliomas with increases in clinical invasion as assessed by magnetic resonance imaging. This would be consistent with CB playing an active role in glioma invasion. In terms of other tumors, Kos *et al.* [29] recently reported that high CB levels are a significant prognostic indicator for relapse of breast cancer and decreased disease-free survival rate in head and neck cancers. High CB levels are also correlated with shorter overall survival of melanoma patients [29]. We predict that high levels of CB will be a prognostic indicator in many cancers.

2.2. CB expression at the invasive edge

The expression of CB is often increased specifically at the invasive edges of tumors. In prostate cancer, increases in CB mRNA can be observed in tumor cells at the invasive edges by *in situ* hybridization [30]. Similarly, the levels of CB protein (as determined by immunohistochemical analyses) are increased at the invasive edges of bladder, colon, and prostate carcinomas and in infiltrating glioblastoma cells [24,26, 31-33]. Crucial to this enzyme playing a functional role at the invasive edge is that the protein staining at the invasive edge of human colon and breast carcinomas and at the infiltrating edge of glioblastomas represents active CB (as determined by enzymatic assays of microdissected specimens) [34,35]. These *in vivo* studies demonstrate an increase in CB expression in several different tumor types as well as an increased localization of active forms of CB in the region where the invading tumors are degrading the basement membrane. An inverse correlation between staining for CB and type IV collagen is present at the invading edges of bladder tumors and for CB and laminin in pulmonary adenocarcinomas [36; D.W. Visscher, M. Sameni, and B.F. Sloane, unpublished results]. This would be consistent with a

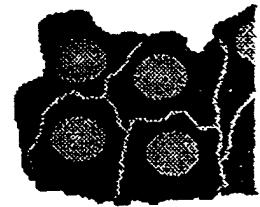
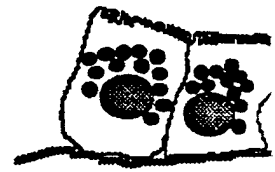


Figure 2. Schematic representation of a cell in normal colonic carcinoma. In normal colonic carcinoma, the CB staining remains vesicular, but the staining is seen in later stages of colon carcinoma.

functional role for CB in *in vitro* [7,9]. The major role of CB suggests that interaction with CB is important for expression.

3. Cathepsin B Localization

3.1. Change in CB localization

Location, location, location, location seems to be the key to progression. Enzymes that are involved in cell death should be located at the site of the cell. In this case for CB, a lysosome is at one pole of the nucleus. CB staining would be granular. Studies of human tumor cells have shown that CB staining is vesicular and in the apical

es Stage [25].

High Staining	
0	(0%)
5	(25%)
13	(44%)
8	(72%)

Protein staining for CB is
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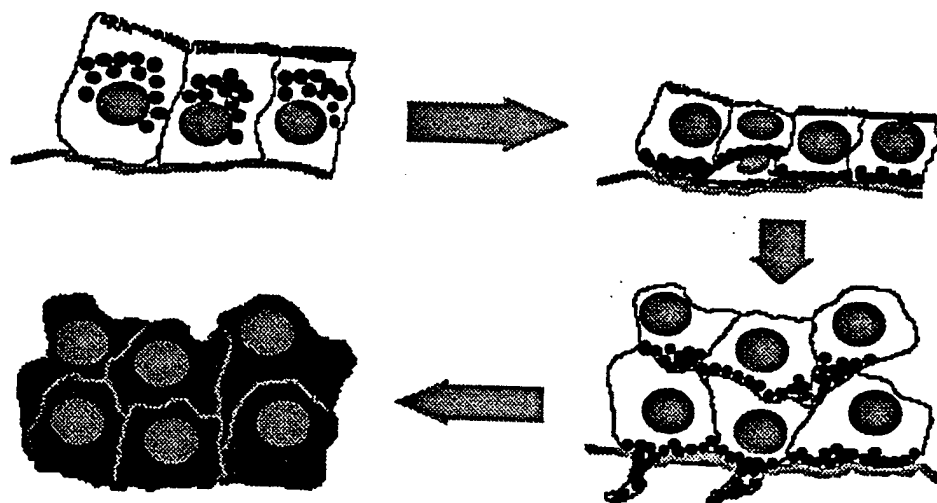


Figure 2. Schematic representation of the altered subcellular localization of CB in the progression of colon carcinoma. In normal colonic epithelial cells (top left), vesicular staining for CB is seen in the apical portion of the cell. In late adenoma to early carcinoma (top right and bottom right, respectively), CB staining remains vesicular, but the staining is now redistributed along the inner basal surface of the cell adjacent to the basement membrane. At this stage, basement membrane dissolution occurs. Diffuse cytoplasmic staining is seen in later stages of colon carcinoma, Dukes' C and D (bottom left). [25]

functional role for CB in degrading these extracellular matrix components *in vivo* as well as *in vitro* [7,9]. The markedly increased expression of CB at the tumor/stromal interface suggests that interactions between the tumor and surrounding stroma may upregulate CB expression.

3. Cathepsin B Localization in Tumor Cells

3.1. Change in CB localization in colon carcinoma cells

Location, location, location! Just as location is critically important to property values, location seems to be critically important to the involvement of CB in malignant progression. Enzymes that participate in degradation of the extracellular matrix by tumor cells should be located at or near the surface of tumor cells, or secreted from tumor cells. Is this the case for CB, a lysosomal cysteine protease? The normal localization of lysosomes is at one pole of the nucleus in the region of the microtubule organizing center and therefore CB staining would be predicted to be in this region. However, immunohistochemical studies of human tumors reveal a different pattern for CB staining. In 69 colorectal carcinomas that were analyzed, CB staining undergoes a redistribution in parallel with the malignant of the tumors (figure 2) [25]. In normal colonic epithelium, CB staining is vesicular and in the apical region of these polarized cells. At some point during progression

to late adenoma and early carcinoma, CB staining moves to the inner basal surface of the plasma membrane immediately adjacent to the underlying basement membrane. The CB staining in late adenomas/early carcinomas is vesicular. Changes in localization of CB seem to precede the increase in CB protein, thus indicating that alterations in trafficking of CB are independent of increases in expression. In later stages of colorectal cancer (Dukes' stages C and D), a diffuse cytoplasmic staining is sometimes observed for CB. This diffuse cytoplasmic staining may be due to a truncated form of proCB that would be missing the signal peptide and therefore targeted to the cytoplasm (as described in section 1.2), but this has not yet been confirmed.

3.2. Localization of CB at the cell periphery and beyond

Altered subcellular distribution of CB is seen in preneoplastic variants of the MCF10 human breast epithelial cell line. MCF10A cells transfected with the c-H-ras oncogene gave rise to the MCF10AneoT cell line, a line with characteristics of atypical breast epithelial stem cells. In nude mice, the neoT cells form hyperplastic lesions and *in situ* carcinomas [37]. In the parental 10A cell line, the subcellular localization of CB is perinuclear [17]. In neoT cells, staining for CB is observed in the perinuclear region as well as in the cell periphery, including in cell processes [17]. Extracellularly, staining for CB is located to patches on the basal surface of neoT cells, but is not present on the extracellular surface of parental 10A cells [38,39]. Similar, but more pronounced, alterations in the distribution of CB can be observed in human breast carcinoma cell lines and glioma cell lines [27,38]. In these cells, we have shown that CB staining is concentrated in cell processes [24], in regions resembling focal adhesions. Staining for CB does colocalize with vinculin, thus confirming that CB is localized in focal adhesions. Three dimensional reconstruction of confocal sections of these cells indicate that the CB in the focal adhesions is at the inner aspect of the basal surface of the cells. As this is the region where the cell would be in contact with the extracellular matrix, our observations would be consistent with a functional role for CB at cell attachment sites. We have established in human breast carcinoma cells that CB staining at attachment sites can be increased by incubation with tumor necrosis factor α [M. Sameni and B.F. Sloane, unpublished data]. In motile cancer cells, CB staining also is concentrated in this case in the "tail end" of the cells where they are still attached to matrix. The concentration of CB in the "tail end" of the cell suggest a role for CB in cell detachment and thus in cell motility. In a recent review by Bretscher [40], polarized endocytosis of cell adhesion proteins is suggested as mechanism for cell migration. Bretscher suggests that the principal function of polarized endocytosis "may be to assist in the transfer of a cell's feet from its rear up to its front." [40] We hypothesize that CB moves to the trailing edge of these carcinoma cells, degrades the underlying matrix or proteins/receptors involved in cell attachment to the matrix so that the cells can then move forward.

In human breast carcinoma cells and human glioma cells grown on fluorescein-labeled laminin substrate, CB can be seen to be localized on the cell surface in the area where laminin is degraded [41]. A specific inhibitor for CB, CAO74, can reduce the laminin degradation [M. Sameni and B.F. Sloane, unpublished results]. To date, our results are qualitative; quantitative studies are ongoing. Nonetheless, CB localization on the

surface of tumor cell line suggests a possible functional role for CB.

3.3. Binding of CB to the extracellular matrix

The association of CB with the extracellular matrix associated binding protein (ECM-BP) has been purified in our laboratory. This protein can be inhibited by unlabeled mannose or preimmune mannose 6-phosphate. This protein has been used to probe a plasma membrane protein specifically to a 70 kD protein which can localize in extracellular matrix during invasive processes that occur in cancer cells.

3.4. Secretion of CB from cells

Active CB has been found in the conditioned media of sublines of B16 murine melanoma, colorectal carcinoma cell lines, and human breast carcinoma cell lines. Interestingly, an inverse relationship between CB secretion and invasiveness of CB in these cell lines has been observed, whereas the more invasive cell lines show less CB activity. Perhaps the release of CB is related to the movement through the basement membrane.

4. Does Cathepsin B Play a Role in Cell Migration?

4.1. CB expression in invasive cells

Angiogenesis, the process by which new blood vessels form from existing microvasculature, is a key step in tumor invasion and metastasis. Staining for CB is observed in the perinuclear region of existing microvasculature. This has been demonstrated by *in situ* hybridization of human glioma cells, *in vitro*, when human endothelial cells are grown on Matrigel, microvessels

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CB localization on the

surface of tumor cell lines and specifically in areas of laminin degradation indicate a possible functional role for this enzyme in tumor cell invasion.

3.3. Binding of CB to the cell surface

The association of CB with the cell surface led us to look for a plasma membrane-associated binding protein for CB. Biotinylated recombinant proCB (rproCB) expressed and purified in our laboratory [42] has been shown to bind to intact cells. The binding can be inhibited by unlabeled rproCB and an anti-CB IgG, but not by mannose 6-phosphate, mannose or preimmune serum. These results indicate that rproCB is not bound to mannose 6-phosphate receptors recycled via the cell surface. Biotinylated rproCB has been used to probe a plasma membrane extract run on a SDS-PAGE gel and shown to bind specifically to a 70 kD protein [43]. This 70 kD protein may represent a novel binding protein which can localize CB to discrete regions on the cell surface where it may function in extracellular matrix degradation. Thus this binding protein may be important to local invasive processes that occur during tumor progression.

3.4. Secretion of CB from tumor cells

Active CB has been found to be secreted from the most invasive or metastatic sublines of B16 murine melanoma, MCF-10 human breast epithelial cells, KM12 human colorectal carcinoma cell lines and glioma cell lines [44,45]. MCF-7 and BT20 human breast carcinoma cell lines also secrete proCB as well as mature active CB [46]. Interestingly, an inverse correlation is found between intercellular and extracellular activity of CB in these cell lines. MCF-7 has high intercellular and low extracellular activity, whereas the more invasive BT20 cell line has low intercellular but high extracellular activity. Perhaps the more invasive tumor cells are secreting active CB to facilitate movement through the basement membrane.

4. Does Cathepsin B Play a Role in Tumor Cell invasion?

4.1. CB expression in invading endothelial cells

Angiogenesis, the process of neovessel formation, parallels functionally tumor cell invasion and metastasis [47]. In human prostate and breast carcinomas and gliomas, CB staining is observed in endothelial cells of neovessels, but not in the endothelial cells of pre-existing microvasculature [26, 48; T.T. Lah, personal communication]. Recently, we have demonstrated by *in situ* hybridization increases in CB expression in the endothelial cells of neovessels of human gastric tumors [W.P. Ren and B.F. Sloane, unpublished data]. *In vitro*, when human endothelial cells isolated from the breast microvasculature are grown on Matrigel, microvessels form which can be imaged solely by their intense staining for CB

[D. Keppler and B.F. Sloane, unpublished data]. These results indicate that CB may be involved in endothelial cell invasion induced by tumors as well as in tumor cell invasion.

4.2. Inhibition of tumor cell invasion by CB inhibitors

Studies with CB inhibitors provide direct evidence that CB participates in tumor invasion. E-64, a cysteine protease inhibitor, and the propeptide of CB inhibit invasion of the murine Lewis lung carcinoma cell line, H59, *in vitro*. E-64 also blocks liver metastasis by these cells *in vivo* [49]. *In vitro*, two classes of CB inhibitors (lipophilic inhibitors and vinyl sulfones) reduce invasion of human glioma cells in Matrigel invasion assays and reduce infiltration of these cells into normal rat brain aggregates in spheroid confrontation assays [50]. RAT-1 fibroblasts when transfected with activated *rac* become highly invasive and their invasion through Matrigel can be reduced with CB-selective synthetic cysteine protease inhibitors [D. Kim, personal communication]. Such studies establish that CB is involved in invasion by some transfected and tumor cell lines. The ability of CB inhibitors to decrease this invasion indicates their potential usefulness as therapeutic agents.

5. Conclusions

CB mRNA, protein, and activity in human and rodent tumors are elevated in parallel with increasing malignancy [16,51,52]. The redistribution of CB within tumor cells as well as the increased expression of CB in tumor cells adjacent to the extracellular matrix suggest that there are mechanisms for mobilization of CB to regions of tumor cell invasion. *In vitro* and *in vivo* studies have shown that CB inhibitors can reduce the invasive and metastatic capabilities of tumor cells. The effect of the CB inhibitors on tumor invasion could be direct due to inhibition of extracellular matrix proteolysis by CB or indirect due to inhibition of activation of other proteases by CB. The data presented in this chapter support a functional role for CB in tumor cell invasion and perhaps in an associated angiogenic process. Changes in the expression and localization of CB also occur in disease states such as arthritis, inflammation and parasitic infection and developmental processes. Thus, understanding the regulatory mechanisms underlying the increases in expression and altered localization of CB in human tumors may well have implications to a broad spectrum of biological and pathological processes.

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Grants CA 36481 and 56586.
a grant from the Elsa U. Pardee
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